VITAMINS AND HORMONES

VOLUME VI

VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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VOLUME VI

With Cumulative Subject Index of Vols. I–V

1948
ACADEMIC PRESS INC. PUBLISHERS
NEW YORK

125 EAST 23RD STREET
NEW YORK 10, N. Y.

PRINTED IN THE UNITED STATES OF AMERICA

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EDITORS' PREFACE

The favorable reception of preceding volumes makes it clear that *Vitamins and Hormones* continues to serve a valuable function. Indeed, both the wide scattering of the literature and its ever increasing volume are only too obvious to workers in the field. Some aspects of the breadth of this field are shown by three of the articles in this volume which bring out the importance of the vitamins as pharmacological agents, as plant hormones, and as products of industrial microbiology.

A cumulative subject index of Volumes I through V is included here. The compilation of this valuable index by Dr. Martha Sinai should make the series considerably more useful as a reference work.

It is regretted that unavoidable difficulties should have delayed the appearance of this volume by some four months. With the following volume it is expected that a return will be made to the normal schedule.

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CONTENTS

Nutritional Requirements of the Cotton Rat and Hamster

By B. S. Schweigert, *Division of Biochemistry and Nutrition, American Meat Institute Foundation, University of Chicago, Chicago, Illinois*

Vitamins as Pharmacologic Agents

By Hans Molitor and Gladys A. Emerson, *Merck Institute for Therapeutic Research, Rahway, New Jersey*

The Assessment of Human Nutriture

By H. M. Sinclair, *Wellcome Laboratory of Human Nutrition, University of Oxford*

Vitamins in Microorganisms—Distribution and Quantitative Synthesis

By J. M. Van Lanen, *Hiram Walker and Sons, Inc., and Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Illinois,* and Fred W. Tanner, Jr., *Chas. Pfizer and Co., Inc., Brooklyn, N. Y.*

The B Vitamins as Plant Hormones

By James Bonner and Harriet Bonner, *William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California*

The Influence of the Adrenal Cortex on the Metabolism of Water and Electrolytes

By Edward C. Kendall, *Division of Biochemistry, Mayo Foundation, Rochester, Minnesota*

The Chemistry and Biological Action of Pteroylglutamic Acid and Related Compounds

By BRIAN L. HUTCHINGS AND JOHN H. MOWAT

Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

CONTENTS

I. Introduction

In an excellent review Pfiffner and Hogan (1946) correlated the available pertinent data on what they termed the "Newer Hematopoetic Factors of the Vitamin B Complex." During the ensuing two years much of the information regarding the isolation, degradation and synthesis of the factors variously known as vitamin B_c, vitamin B_c conjugate, liver *Lactobacillus casei* factor, fermentation *L. casei* factor and rhizopterin (S.L.R. factor) has been published and thus it is now possible to bring order to the confusing terminology and to correlate and present the data on a rational chemical basis. For the information prior to 1945, the reader is referred to the above mentioned review.

II. Isolation of Vitamin B_C

Pfiffner *et al.* (1943) reported the isolation of a compound which was active for *Lactobacillus casei* and which promoted growth and hemo-

globin formation in the chick. This factor was identical to that of Hogan and Parrott (1939, 1940) and was designated "vitamin B_c" because of its relation to chick nutrition. In a later publication (Pfiffner *et al.*, 1947) the isolation procedure and certain chemical properties of the compound were described.

After autolysis or suitable enzymatic digestion depending upon the tissue used as a source material, an aqueous extract was prepared. The active compound was thence successively adsorbed on and eluted from Amberlite IR-4, Super Filtrol, and Norite SG-11. The eluant in each case was either aqueous ammonia or a 5% ammonia−50% ethanol mixture. After removal of the ethanol and suitable concentration, the aqueous solution was extracted at pH 5.6 with butanol to remove non-acidic impurities. The active compound was then extracted into butanol at pH 3.0. After concentrating and cooling, the precipitated compound was redissolved in 90% methanol and then precipitated as the barium salt. The water soluble barium salts were treated with a solution of zinc acetate and the insoluble zinc salts collected. After decomposition of the zinc salt followed by reprecipitation as the barium salt, the free acid could be crystallized from hot water.

By this procedure or slight modifications thereof, crystals that exhibited identical properties were isolated from hog and horse liver and from yeast extract (Difco).

The pale yellow crystals exhibited no melting point but darkened and charred from 250°. The extremes of the analytical values were as follows:

C, 50.5–53.06; H, 4.05–5.00; N, 18.91–22.39 (Kjeldahl), 19.91–22.04 (Dumas).

Molecular weight determinations on the free acid gave values of 370 and 377 and on the dimethyl ester values of 463, 407, and 394 were obtained. Various salts and derivatives of the compound were prepared such as the diethyl ester, dimethyl ester dihydrochloride, diamide, silver salt, and disodium salt.

III. Isolation of Vitamin B_c Conjugate

Yeast and yeast extracts are quite inactive when assayed microbiologically but are potent sources of antianemia activity when assayed by chicks. The isolation of the active compound present in such crude materials was announced by Pfiffner *et al.* (1945). The compound possessed approximately 0.4% activity for *L. casei* and 0.2% activity for *Streptococcus faecalis* R. The ultraviolet absorption spectra of the conjugate were similar to the spectra of vitamin B_c with the difference

that the $E^{1\%}_{1\,cm}$ values were only 35.7% as great. The antianemic activity of the conjugate for the chick corresponded to the vitamin B_c content of the compound as calculated from the ultraviolet absorption data. For further information the review of Pfiffner and Hogan (1946) should be consulted.

Only fragmentary data on the actual method of isolation of the conjugate has appeared. Pfiffner *et al.* (1947) have indicated that the preliminary isolation steps consisted of two consecutive adsorptions and elutions from Norite SG-11. No additional data have appeared.

IV. Isolation of the Liver *Lactobacillus casei* Factor

Stokstad (1943) announced the isolation of a compound from liver and from yeast. In a later publication (Stokstad, Hutchings, and SubbaRow, 1946, 1948) the detailed procedure for the isolation of the liver compound was outlined. The source material was a dried 85% ethanol precipitate of an aqueous extract of liver. The active compound was adsorbed on and eluted from Norite A and Super Filtrol. This was followed by precipitation with barium and ethanol, and esterification of the precipitate, after drying, with methanolic hydrogen chloride. After esterification the solution was concentrated nearly to dryness and then dissolved in water and the active compound extracted into butanol. After removal of the butanol, the residue was dissolved in methanol and chromatographed on Super Filtrol. The column was developed with aqueous acetone and the 70% aqueous acetone eluates contained the activity. On removal of the acetone, the ester precipitated and was collected. Residual pigments were removed with small amounts of cold methanol. After reprecipitation from methanol, the purity of the compound could not be increased.

Combustion data for two samples were as follows:

Sample #1, C, 53.0; H, 4.6; N, 21.2
Sample #2, C, 53.1; H, 5.1; N, 20.5

After hydrolysis of the ester the free acid was crystallized from water. The extinction coefficients in 0.1 N sodium hydroxide at points of maxima were $E^{1\%}_{1\,cm}$: 255 mμ, 565; 282 mμ, 350; 365 mμ, 195.

The compounds isolated by Pfiffner and coworkers from liver and yeast and the compound isolated by Stokstad and associates from liver were found to be essential for the growth of *Lactobacillus casei, Streptococcus faecalis* R. and the chick when compared under similar conditions. The ultraviolet absorption data and the analytical data were essentially in agreement and it was correctly deduced that the compounds were identical.

V. Isolation of the Fermentation *Lactobacillus casei* Factor

The isolation of a compound with different microbiological properties was reported by Hutchings *et al.* (1944). Later the source material was described and an outline of the method of isolation was presented (Hutchings, Stokstad, Bohonos, *et al.* (1946, 1948). The source material was a filtrate obtained from the aerobic growth of an organism classified as *Corynebacterium* sp. (Hutchings and Sloane, unpublished data). After removal of the bacterial cells the active compound was adsorbed on charcoal at pH 3.0 followed by elution with ethanolic ammonium hydroxide. The compound was then esterified, extracted into butanol, and fractionated from methanol. The purified ester was saponified with barium hydroxide. Residual extraneous pigments were removed by absorption with Florisil and precipitation of the compound as the free acid yielded essentially a pure product.

The compound could be crystallized only in the presence of electrolytes. If the electrolytes were absent, gels were invariably formed. Under the defined conditions it was possible to crystallize the compound as the free acid, barium salt, and methyl ester.

Consistent analytical data for the fermentation product were not obtained. The following values are representative:

C, 47.7—48.6; H, 4.4–4.8; N, 15.5–16.2

The fermentation compound is markedly more soluble as the free acid or as the methyl ester in various solvents than is the compound isolated from liver. The distinguishing characteristic of the compound is its relative activity for *Lactobacillus casei* and *Streptococcus faecalis* R. The fermentation compound is 60–80% as active as the liver compound for *L. casei* but only 4–6% as active for *S. faecalis* R.

Hutchings, Oleson, and Stokstad (1946) in a study of its activity for the chick reported data indicating that on a molar basis it was as active as the liver compound. The fermentation compound has also been reported as being efficacious in the treatment of vitamin M deficiency in the monkey (Day *et al.*, 1945) and in relieving the sulfonamide-induced deficiency in the rat (Daft and Sebrell, 1943).

VI. Isolation of Rhizopterin (S.L.R. Factor)

The isolation of a factor highly active for *Streptococcus lactis* R. but essentially inactive for *Lactobacillus casei* was reported by Keresztesy *et al.* (1943). Recently, the isolation, degradation, and synthesis of this compound has been described.

When natural materials are subjected to differential assay with *S. lactis* R. and *L. casei*, certain materials are much more highly active for *S. lactis* R. than for *L. casei*. The source material used by Rickes *et al.* (1947) exhibited this property to a marked degree. This material was a charcoal adsorbate derived from the purification of liquors of *Rhizopus nigricans* fumaric acid fermentation.

The crystalline compound was obtained by the following purification steps: elution of the charcoal adsorbate, readsorption on Norite A and elution, adsorption on Fullers earth and elution, precipitation at pH 7.0, chromatographic adsorption on alumina, and crystallization as the free acid or ammonium salt. The isolated compound was highly active for *S. lactis* R. (0.000034γ/ml. for half-maximum growth), but inactive for *L. casei*, the chick and the rat.

Analytical data for the free acid suggested the formula $C_{15}H_{12}N_6O_4$. Potentiometric titration data indicated the substance was a dibasic acid with an equivalent weight calculated to be 167.5 or a molecular weight of 335. The compound was essentially insoluble in water and in the common organic solvents but dissolved readily in acid or alkali.

The ultraviolet absorption spectra of the compound had points of similarity to the spectra of xanthopterin. The ultraviolet absorption data and the physical properties of the compound suggested the presence of a pteridine nucleus. On this basis the name rhizopterin was suggested.

Wolf *et al.* (1947) in a refinement of the isolation technique used luteo ethylenediaminocobaltic chloride to precipitate rhizopterin from crude preparations. After crystallization from water, the compound was converted to rhizopterin by treatment with dilute acetic acid.

VII. Degradation of Vitamin B_C

Wittle *et al.* (1947) reported the oxidative degradation of vitamin B_c. When the compound or its dimethyl ester was oxidized with either permanganate or chloric acid, a fluorescent microcrystalline solid was obtained. The compound contained a carboxyl group as evidenced by the formation of a methyl ester. Analytical values for the free acid varied. The possible empirical formulations were $C_8H_7O_3N_5$, $C_9H_6O_4N_6$, and $C_7H_5O_3N_5$. Further oxidation with chloric acid yielded guanidine. The formation of guanidine indicated the presence of a 2-aminopyrimidine ring. This evidence taken in conjunction with the analytical values and the absorption data strengthened the supposition that the oxidation product was a pterin.

A study of the ultraviolet absorption data of various pterins eliminated from consideration those having a hydroxyl group at position 6 or 7.

The compound that most nearly resembled the unknown acid was synthesized by condensing ethyl α,β-diketobutyrate with 2,4,5-triamino-6-hydroxypyrimidine. The structure assigned was that of 2-amino-4-hydroxy-6-carboxy-7-methylpteridine on the basis that the more reactive α-carbonyl in ethyl α,β-diketobutyrate condensed with the more basic 5-amino group in 2,4,5-triamino-6-hydroxypyrimidine. However, the ultraviolet absorption spectrum of the synthetic pteridine in 0.1 *N* acid was significantly different from that of the unknown compound. Furthermore, 2-amino-4-hydroxy-6-carboxy-7-methylpteridine on treatment with alkaline permanganate yielded the corresponding 6,7-dicarboxypteridine whereas the unknown acid was unchanged by this treatment. Subsequent disclosure by Angier *et al.* (1946) and Mowat, Boothe, *et al.* (1948) of their structural work indicated that the unknown acid was 2-amino-4-hydroxypteridine-6-carboxylic acid.

Ethyl acetate extraction of the chloric acid oxidized solution yielded a compound melting at 177–179°. Molecular weight determination and analytical values indicated that the compound had the formulation of $C_{12}H_{12}O_5N_2Cl_2$. A diester could be prepared, $C_{14}H_{16}O_5N_2Cl_2$, with either methanolic hydrogen chloride or diazomethane. If a sodium bromate-hydrobromic acid solution was used an analogous compound, $C_{12}H_{12}O_5N_2Br_2$, melting at 205–208° was obtained. When either of these compounds was heated at 210–240°, a decomposition took place and the products distilled from the melt. The compounds were separated by crystallization from dilute methanol. The soluble fraction, after crystallization from acetone, was identified by mixed melting points as *dl*-pyrrolidonecarboxylic acid.

The methanol insoluble fraction melted at 290–293° and from analysis the formula $C_7H_5O_2NCl_2$ was deduced. The disclosure of Angier *et al.* (1946) on the structure of pteroylglutamic acid indicated that the C_{12} dichloro compound should be 3,5-dichloro-4-aminobenzoylglutamic acid and the C_7 compound 3,5-dichloro-4-aminobenzoic acid. The identity of the two products was proved by comparison with synthetic samples of the above compounds.

Vitamin B_c can be readily reduced in 0.1 *N* sodium hydroxide solution over platinum oxide or palladium to yield the dihydrovitamin (O'Dell *et al.*, 1947). During the reduction the characteristic yellow color of the compound is discharged. The ultraviolet absorption maxima in 0.1 *N* sodium hydroxide solution at 255 mμ, 282 mμ, and 365 mμ are destroyed and a new maximum at 284 mμ appears. If the reduction is carried out in glacial acetic acid over platinum, 2 moles of hydrogen are absorbed and the tetrahydro compound is formed. The ultraviolet absorption spectra of this compound in 0.1 *N* alkali are essentially similar to the

spectra of the dihydro derivative. Both reduced compounds on treatment with oxygen are oxidized to form the original compound.

Since O'Dell and coworkers could not readily hydrogenate leucopterin or isoxanthopterin using conditions similar to those for the reduction of vitamin B_c to the dihydro derivative, and since xanthopterin readily adds 1 mole of hydrogen, it was postulated by them that the 7,8-double bond in vitamin B_c (pteroylglutamic acid) is the readily reducible bond.

This ease of oxidation and reduction offers a possible role for pteroylglutamic acid in hydrogen transport in the oxidation-reduction enzyme systems.

VIII. Degradation of Vitamin B_C Conjugate

Ultraviolet absorption data of Pfiffner *et al.* (1945) indicated vitamin B_c conjugate to consist of vitamin B_c linked to an ultraviolet-transparent nitrogenous side chain. In later studies Pfiffner *et al.* (1946) reported that acid hydrolysis of the conjugate liberated 7 moles of glutamic acid. The conjugate is, therefore, vitamin B_c plus 6 additional moles of glutamic acid.

IX. Degradation of the Fermentation *Lactobacillus casei* Factor

The degradative studies leading to the elucidation of the structure of the liver *L. casei* factor and of the fermentation compound were carried out on the fermentation product.

Stokstad, Hutchings, Mowat, *et al.* (1946, 1948) described the degradation of the fermentation compound by anaerobic and aerobic alkaline hydrolysis and by acid hydrolysis.

Acid or alkaline hydrolysis liberated a fluorescent pigment and a diazotizable aromatic amine that could be estimated by the method of Bratton and Marshall (1939). The liberation of amine was most rapid in alkaline solution and oxygen was necessary for the cleavage. When the reaction was carried out anaerobically there was no change in the absorption spectrum and only small amounts of aromatic amine or fluorescent pigment were produced. However, two moles of α-amino acid nitrogen were liberated and because of its precipitability with barium and ethanol the formation of an aminodicarboxylic acid was suggested. The microbiological activity of the original compound had been significantly changed by the anaerobic hydrolysis. The ratio of activity for *L. casei* and *S. faecalis* R. was nearly unity. However, the compound resulting from alkaline hydrolysis was only 58% as active for *L. casei* and 57% as active for *S. faecalis* R. as was the compound isolated from liver. A racemic product was indicated and later, by comparison to a synthetic

product, the anaerobic alkaline hydrolysis product was shown to be the *dl*-liver compound.

The fluorescent pigment formed on aerobic alkaline hydrolysis analyzed for $C_7H_5N_5O_3$. The presence of a 2-aminopyrimidine group was suggested on the basis of the formation of guanidine on oxidation with chlorine water. The compound contained two acid groups with pKa values of 3.9 and 7.7. Decarboxylation of the compound yielded a new fluorescent monobasic acid with a pKa of 8.0. The decarboxylation data demonstrated the presence of a carboxylic and an enolic group in the fluorescent pigment derived from aerobic hydrolysis of the fermentation compound.

The empirical formula, ultraviolet absorption, titration data, and formation of guanidine suggested a 2-aminopteridine containing an enolic group and carboxyl group. Mowat, Boothe, *et al.* (1946, 1948) by synthesizing various pteridines showed the decarboxylated compound to be identical, on the basis of its ultraviolet absorption spectrum, with a synthetic pteridine of known structure, 2-amino-4-hydroxypteridine, prepared from 2,4,5-triamino-6-hydroxypyrimidine (Traube, 1900) and glyoxal.

These results suggested that the dibasic acid was probably a 2-amino-4-hydroxypteridine-6-(or 7) carboxylic acid. The truth of this assumption was demonstrated by the synthesis of the dibasic acid, first, by the chlorination and reduction of the previously known isoxanthopterin carboxylic acid (2-amino-4,7-dihydroxypteridine-6-carboxylic acid) (Purrmann, 1941) whereby the 7-hydroxy group was replaced by hydrogen and second, by condensing 2,4,5-triamino-6-hydroxypyrimidine with ethyl-α-bromo-β,β-diethoxypropionate in the presence of silver carbonate. These syntheses established the nature of the ring system and the positions of the 2-amino group and the 4-hydroxy group. The location of the carboxyl group, although very probably in the 6-position, was not rigorously established, since the structures of xanthopterin and isoxanthopterin carboxylic acid postulated by Purrmann (1940, 1941) were still open to question. Final proof that the carboxyl group of the dibasic acid was indeed in the 6-position was obtained from another pteridine degradation product of the fermentation *L. casei* factor.

Anaerobic acid hydrolysis (Stokstad, Hutchings, Mowat, *et al.*, 1946, 1948) of the fermentation compound yielded a fluorescent pigment and an aromatic amine. Butanol extraction at pH 7.0 of the hydrolyzate removed a pteridine monobasic acid. Crystallization of this compound from alkali produced an analytical product which by comparison to a synthetic pteridine (Mowat, Boothe, *et al.*, 1946, 1948) established its identity as 2-amino-4-hydroxy-6-methylpteridine. This latter compound

was synthesized by reacting 2,4,5-triamino-6-hydroxypyrimidine with methyl γ,γ-dimethoxyacetoacetate to give 2-amino-4-hydroxypteridine-6-acetic acid (which could be oxidized with hot alkaline potassium permanganate solution to 2-amino-4-hydroxypteridine-6-carboxylic acid). Decarboxylation of the pteridineacetic acid gave the desired 2-amino-4-hydroxy-6-methylpteridine identical with that obtained from the fermentation *L. casei* factor. Using a reaction analogous to that carried out by Weijlard *et al.* (1945) in their degradation of lumazines, the 6-methylpteridine was reacted with 4.2 *N* sodium hydroxide solution at 170°C. for 20 hours. The product, 2-amino-5-methylpyrazine-3-carboxylic acid, was isolated and decarboxylated to give 2-amino-5-methylpyrazine which was identical with a known sample prepared from 2,5-dimethylpyrazine. The formation of 2-amino-5-methylpyrazine provides conclusive proof that the pteridine degradation products of the *L. casei* factor(s) are substituted in the 6-position.

Since this review is primarily concerned with the chemical and biological characteristics of the *L. casei* factors, no attempt will be made to cover the field of pteridine chemistry *per se*. The reader who is interested in the earlier work in this field is referred to an excellent review of the chemistry of pteridines by Gates (1947) covering the published data up to 1947, as well as to a number of more recent publications (Elion and Hitchings, 1947; Cain *et al.*, 1946; Mallette *et al.*, 1947; Karrer *et al.*, 1947; Petering and Weisblat, 1947).

The aromatic amine fraction was obtained by hydrolyzing the fermentation compound anaerobically with alkali to yield the *dl*-liver *L. casei* factor (Stokstad, Hutchings, Mowat, *et al.*, 1946, 1948). This compound was then hydrolyzed aerobically with alkali and the resulting pteridines removed by precipitation with silver at pH 3.0. The aromatic amine was then precipitated with barium and alcohol to obtain a product analyzing for 2.1 atoms of total nitrogen for each atom of aromatic amine nitrogen. Subsequent hydrolysis with 2.0 *N* sulfuric acid liberated 45% of the nitrogen as α-amino acid nitrogen. From such hydrolyzates *p*-aminobenzoic acid was isolated and identified by mixed melting points and by its microbiological activity for *Clostridium acetobutylicium* and *Acetobacter suboxydans*.

Further degradative studies on the fermentation factor by Hutchings, Stokstad, Mowat, *et al.* (1946, 1948) made possible the probable structural formulation of the liver *L. casei* factor.

When the fermentation compound was autoclaved at pH 4.0 in aqueous solution, extensive biological inactivation occurred. Concentration of the clarified solution followed by solvent extraction and sublimation yielded a compound, m.p. 147–148°, with an empirical formula

of $C_5H_7O_3N$. Titration data were indicative of the presence of a carboxylic acid group and the equivalent weight was found to be 129.2. Alkaline hydrolysis converted the nitrogen into α-amino acid nitrogen. Microbiological assay of the hydrolyzates demonstrated the formation of glutamic acid. When the unknown was compared to an authentic sample of *l*-pyrrolidonecarboxylic acid, the two were found to be identical.

Sulfurous acid hydrolysis at room temperature of the fermentation compound gave rise to a pteridine and an aromatic amine fraction. The pteridine fraction reacted readily with typical aldehyde reagents. The analytical values for the phenylhydrazone suggested the presence of a pteridine carboxaldehyde.

If the freshly cleaved aldehyde was allowed to stand in dilute alkali anaerobically, the compound underwent a dismutation to yield approximately equivalent amounts of compounds identified as 2-amino-4-hydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxy-6-methylpteridine. The former compound was identified by a comparison of its ultraviolet absorption spectrum with an authentic sample of 2-amino-4-hydroxypteridine-6-carboxylic acid. The methylpteridine was identified by analysis and comparison of its ultraviolet and infrared absorption spectra to those of a known sample of 2-amino-4-hydroxy-6-methylpteridine.

The aromatic amine fraction could not be crystallized but satisfactory analytical data were obtained on the barium salt. Of the nitrogen, 25% was present as aromatic amine nitrogen as measured by the method of Bratton and Marshall. The remaining nitrogen could by acid or alkaline hydrolysis be converted into α-amino acid nitrogen. Microbiological assay of the acid hydrolyzates gave analytical values commensurate with those calculated for 3 moles of glutamic acid. From alkaline hydrolyzates of the amine fraction, *p*-aminobenzoic acid was isolated and characterized by analysis and mixed melting points.

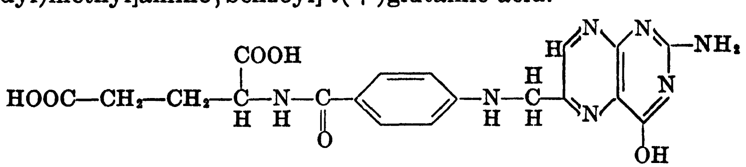
When the fermentation *L. casei* factor was hydrogenated over Raney nickel or palladium on barium sulfate at pH 7.0 and atmospheric pressure, no measurable biological inactivation occurred. However, if the hydrogenation with the palladium-barium sulfate catalyst was carried out at pH 1.0 or 3.0, there was rapid biological inactivation with the simultaneous formation of an aromatic amine. The pteridine moiety was reduced to a compound that exhibited markedly less ultraviolet absorption. An identical cleavage was effected by chemical means, i.e., zinc dust and hydrochloric or sulfuric acids. From such solutions, after reoxidation with manganese dioxide, a pteridine was isolated and characterized as 2-amino-4-hydroxy-6-methylpteridine.

The degradative data on the fermentation *L. casei* factor permitted the following conclusions. The formation of a primary aromatic amine

and a pteridine by aerobic alkaline hydrolysis, acid hydrolysis, sulfite cleavage, and chemical or catalytic reduction made it probable that the point of attachment was through the aromatic amine group to the pteridine. That the bridge at the point of attachment consisted of a single carbon atom was apparent from the formation of either 2-amino-4-hydroxypteridine-6-carboxylic acid or 2-amino-4-hydroxy-6-methylpteridine or a compound that could be converted into them as degradation products in several methods of cleavage. The evidence suggested that the carbon was present as a methylene group. The necessity for oxygen for the alkaline hydrolysis and the reductive formation of the methylpteridine were in accord with this hypothesis. This assumption was further strengthened by the observation that a simple model compound, *N*-benzyl-*p*-aminobenzoic acid (Mowat, Boothe, *et al.*, 1946, 1948), was stable in the presence of hot 1 *N* sodium hydroxide solution in the complete absence of oxygen, but was readily cleaved if oxygen was present, just as in the case of the fermentation *L. casei* factor.

The aromatic amine formed on cleavage with sulfurous acid was a tetrapeptide composed of 3 moles of glutamic acid and 1 mole of *p*-aminobenzoic acid. In degrading the fermentation compound to the *dl*-liver *L. casei* factor, 2 moles of glutamic acid were liberated. Hence, the liver *L. casei* factor contains 1 mole of glutamic acid and the fermentation compound, 3 moles of glutamic acid.

The liver *L. casei* factor is therefore *N*-[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl]-*l*(+)glutamic acid.



X. Degradation of Rhizopterin (S.L.R. Factor)

When a solution of rhizopterin was allowed to stand in dilute alkali, the *S. lactis* R. activity of the solution decreased to one-tenth that of rhizopterin but the *L. casei* activity remained unchanged (Rickes, Trenner, *et al.*, 1947). From such solutions a compound analyzing for $C_{14}H_{10}N_6O_3Na_2$ was isolated. The new compound, termed aporhizopterin, was a weakly dibasic acid, having a calculated equivalent weight of 150.

The new compound exhibited different ultraviolet absorption characteristics than did rhizopterin. In dilute alkali the absorption increases in the region of 280 mμ. In acid solution, the compound exhibits maxima at 250 mμ and 302.5 mμ whereas rhizopterin was characterized by absorp-

tion bands at 252.5 mμ and 322.5 mμ. The spectra of aporhizopterin closely resembled the spectra for vitamin B_c (pteroylglutamic acid), the only difference being in the intensity of the absorption bands. Keresztesy and coworkers concluded that aporhizopterin had the same chromophoric group as vitamin B_c but differed from it in having a smaller mass.

Further degradative data by Wolf *et al.* (1947) led to a complete characterization of rhizopterin. Various derivatives of rhizopterin were prepared (acetyl, methoxyacetyl, phenylacetyl, benzoyl). A molecular weight determination on acetylrhizopterin gave a value of 369 or a molecular weight of 327 for the parent compound. Potentiometric titration of acetyl rhizopterin indicated the presence of a carboxyl group and a weakly acid group.

Acid hydrolysis of rhizopterin liberated formic acid which was characterized as the *p*-bromophenacyl ester. Formic acid was also formed on treatment of rhizopterin with alkali. After acid or alkaline hydrolysis, the product would form a diacetyl derivative.

When the alkaline hydrolysis product (aporhizopterin) was oxidized with potassium chlorate in the presence of excess hydrochloric acid, chloranil and oxaloguanidine were obtained. The former product indicated the presence of a substituted benzenoic nucleus whereas the latter product suggested the presence of a 2-aminopyrimidine ring.

Thermal decomposition of the alkaline hydrolysis product or rhizopterin yielded *p*-aminobenzoic acid characterized as *p*-acetamidobenzoic acid.

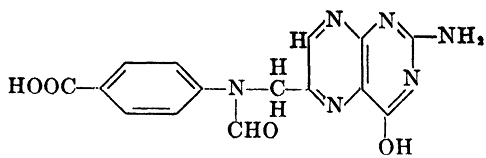
The position of the formyl group was deduced from the fact that oxidation of benzoyl xanthopterin or benzoylrhizopterin formed benzoylguanidine. This evidence when taken in conjunction with the fact that rhizopterin contains one acylatable group and aporhizopterin two acylatable groups locates the formyl group on the nitrogen of the *p*-aminobenzoic acid moiety.

Treatment of rhizopterin with sodium nitrite in concentrated hydrochloric acid solution removed the 2-amino group to form a compound, $C_{15}H_{11}N_5O_5$, designated as desiminorhizopterin. Acid hydrolysis of this product readily removed the formyl group.

When either desiminorhizopterin or aporhizopterin was treated with nitrous acid, a stable nitroso derivative was obtained demonstrating the secondary nature of the *p*-aminobenzoic acid nitrogen.

The formyl, 2-amino-4-hydroxypteridine and *p*-aminobenzoic acid groupings accounted for all of the elements in the molecular formula of rhizopterin. As the analyses for *C*-methyl, *O*-methyl and *N*-methyl were negative, the remaining carbon atom must be present as a bridge.

The proof of structure of rhizopterin was definitely established when the compound was synthesized by reacting formic acid with pteroic acid. Rhizopterin is therefore *p*-[*N*-(2-amino-4-hydroxypyrimido[-4,5-*b*] pyrazin-6-ylmethyl)-formamido]benzoic acid.



Rhizopterin

XI. Synthesis of the Liver *Lactobacillus casei* Factor

Four different methods for the synthesis of the liver *L. casei* factor have been described.

The first, that of Waller *et al.* (1946, 1948) was effected by adding an alcoholic solution of 2,3-dibromopropionaldehyde to a stirred, aqueous solution of 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoyl-*l*(+)glutamic acid while maintaining the reaction mixture at about pH 4.0 by the addition of alkali or by the use of a suitable buffer. The precipitated crude product, containing approximately 15% by weight of biologically active material, was collected and purified.

Similar material has been synthesized by Hultquist *et al.* (1947, 1948) using the following stepwise procedure.

2,3-dibromopropionaldehyde was condensed with pyridine to give the quaternary salt, *N*-[2-formyl-2-bromoethyl]pyridinium bromide. Condensation of this substance with 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride in the presence of potassium iodide yielded the crystalline *N*-[(2-amino-4-hydroxy-6-pteridyl)methyl]pyridinium iodide. This compound was reacted with *p*-aminobenzoyl-*l*(+)glutamic acid in hot ethylene glycol in the presence of sodium methoxide. The reaction mixture, after dilution with water and acidification to pH 3.0, gave a precipitate containing about 25% by weight of biologically active material.

A third method of synthesis, that of Angier *et al.* (1948) involved the condensation of diethyl *N*-(*p*-aminobenzoyl)-*l*(+)glutamate with reductone (2,3-dihydroxyacrylaldehyde) to give diethyl *N*-{*p*-(2,3-dihydroxy-2-ene-propylideneamino)-benzoyl}glutamate. A mixture of this substance and 2,4,5-triamino-6-hydroxypyrimidine, when heated in ethylene glycol at 135° gave a crude product containing about 20% of active material.

In a fourth synthesis, that of Boothe, Waller, *et al.* (1948), 2-amino-4-

hydroxy-6-methylpteridine was treated with bromine in a sealed tube at 155° or with bromine in hot hydrobromic acid to yield a crude bromomethylpteridine. An analogous chloro compound was obtained when the methylpteridine was reacted with sulfuryl chloride in the presence of a trace of benzoyl peroxide.

Condensation of the crude bromomethylpteridine with diethyl *N*-(*p*-aminobenzoyl)-*l*(+)glutamate in hot ethylene glycol yielded a crude product containing about 15% of biologically active material.

Condensation of the crude chloromethylpteridine by the same procedure gave a material containing about 1.0% biological activity.

Purification (Waller *et al.*, 1948) of the materials obtained from each of the four syntheses described above was effected by dissolving the crude reaction product in hot water at a concentration of 0.0002 g. of activity/ml. by the addition of sodium hydroxide to pH 10–12. This solution was adjusted to pH 7.0, cooled and filtered, and the active material was precipitated from the filtrate at pH 3.0. This precipitate was again dissolved in dilute alkali, clarified with charcoal, and reprecipitated at pH 3.0 by the addition of hot dilute acetic acid. This partially purified substance was collected and dissolved in dilute alkali at about 0.002 g./ml. and again clarified with charcoal. The solution was then diluted with hot water and acidified to pH 3.0 with acetic acid. On cooling the active compound crystallized. Further purification was effected by recrystallization from hot water.

Another purification procedure has also been described in an earlier publication (Waller *et al.*, 1946).

The purified products from each of the above syntheses were compared and found to be identical and to possess chemical, physical, and biological properties identical with those of the naturally occurring liver *L. casei* factor.

By the use of *p*-aminobenzoic acid instead of *p*-aminobenzoylglutamic acid in the above syntheses there was obtained [4-{(2-amino-4-hydroxy-6-pteridyl)methyl}-amino]-benzoic acid, the fundamental structural unit common to all of the known *L. casei* factors and rhizopterin. For obvious reasons, the chemical name of this compound is too cumbersome for general usage, and Waller *et al.* (1946, 1948) proposed for it the name "pteroic acid," and for the [4-{(2-amino-4-hydroxy-6-pteridyl)methyl}-amino]-benzoyl radical the name "pteroyl" was suggested. Using this nomenclature, the liver *L. casei* factor becomes pteroylglutamic acid, the fermentation *L. casei* factor becomes pteroyltriglutamic acid, etc. Of course, various modifications may be used to indicate which of several isomers is meant, as for example, pteroyl-γ-glutamyl-γ-glutamylglutamic acid, or pteroyl-α-glutamylglycine, etc.

XII. Synthesis of Pteroylpolyglutamic Acid Compounds

Hutchings, Stokstad, Mowat, *et al.* (1946, 1948) have stated that the so-called fermentation *L. casei* factor contains 3 molecules of glutamic acid in the peptide side chain. It is obvious that these three glutamic acid molecules could be arranged in five different but isomeric linkages. Which of these five possible structures of pteroyltriglutamic acid represented the fermentation *L. casei* factor could be determined either by a difficult stepwise degradation of the natural material or by chemical synthesis of these five possible forms, followed by direct comparison with the natural substance. Although the published record is not yet complete, a recent publication by Boothe, Mowat, *et al.* (1948) indicates that the fermentation *L. casei* factor is probably identical with synthetic pteroyl-γ-glutamyl-γ-glutamylglutamic acid. This substance was synthesized by the following procedure.

The γ-acid chloride of α-ethyl carbobenzoxyglutamate was condensed with diethylglutamate to give triethyl carbobenzoxy-γ-glutamylglutamate. After hydrogenolysis of the carbobenzoxy group, the dipeptide, triethyl-γ-glutamylglutamate hydrochloride, was isolated and again condensed with the γ-acid chloride of α-ethyl carbobenzoxy-glutamate to give tetraethyl carbobenzoxy-γ-glutamyl-γ-glutamylglutamate. After hydrogenolysis of the carbobenzoxy group, the tripeptide was reacted with *p*-nitrobenzoyl chloride. The resulting *p*-nitrobenzoyl-γ-glutamyl-γ-glutamylglutamic acid tetraethyl ester was reduced to the corresponding *p*-aminobenzoyl-γ-glutamyl-γ-glutamylglutamic acid tetraethyl ester. This compound was then coupled with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde by a previously described method (Waller *et al.*, 1946, 1948).

The quantity of pteroyl-γ-glutamyl-γ-glutamylglutamic acid actually obtained was too small to permit complete purification, but biological assay data agreed with the values obtained from the natural fermentation *L. casei* factor.

In the same paper, Boothe and coworkers describe the synthesis and purification of one of the two possible isomers of pteroyldiglutamic acid, namely, pteroyl-γ-glutamylglutamic acid.

The other possible isomer of pteroyldiglutamic acid, pteroyl-α-glutamylglutamic acid, has been described by Mowat, Hutchings, *et al.* (1948). This compound was prepared by nitrobenzoylation and reduction of α-glutamylglutamic acid (Bergmann and Zervas, 1932) and condensation of the resulting *p*-aminobenzoyl-α-glutamylglutamic acid with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde. The purification of this substance is described.

Mowat and associates also describe the synthesis of another of the pteroyltriglutamic acid isomers, namely, pteroyl-α,γ-glutamyldiglutamic acid. This compound was prepared by condensing the γ-acid chloride of carbobenzoxy-α-glutamyldiethylglutamate with diethylglutamate to give the tetraethyl ester of carbobenzoxy-α,γ-glutamyldiglutamic acid. After removal of the carbobenzoxy group by hydrogenolysis, *p*-nitrobenzoylation, and reduction of the nitro compound, the resulting *p*-aminobenzoyl-α,γ-glutamyldiglutamic acid was condensed with 2,4,5-triamino-6-hydroxypyrimidine and 2,3-dibromopropionaldehyde to give assay quantities of the desired pteroyl-α,γ-glutamyldiglutamic acid. Biological assay data indicated that this isomer was not identical with the fermentation *L. casei* factor.

Although the synthesis of the three remaining isomers of pteroyltriglutamic acid has not yet been published, preliminary data available to the authors indicates that these isomers are probably not identical with the fermentation *L. casei* factor.

XIII. Antagonists of Pteroylglutamic Acid and Related Compounds

It has often been noted that the effect of biologically active nutritional factors may be inhibited or even reversed by the administration of a so-called antimetabolite or antagonist.

During the past year a number of publications have appeared which describe substances antagonistic to the action of pteroylglutamic acid and related compounds.

Pteroylaspartic acid (Hutchings, Mowat, *et al.*, 1947) has been shown to be antagonistic to pteroylglutamic acid and its homologues in the nutrition of certain microorganisms, and of the chick. No inhibitory effect was observed in the rat. The "inhibition index" (the ratio of antagonist to metabolite required for one-half maximum inhibition) was found to be 2.0 for pteroic acid, and 50.0 for pteroylglutamic acid, 7.0 for pteroyl-γ-glutamylglutamic acid, and 0.2 for pteroyl-γ-glutamyl-γ-glutamylglutamic acid in assays using *S. faecalis* R. as the test organism.

In the chick assay, about 500 parts of pteroylaspartic acid was found to inhibit the effect of 1.0 part of pteroylglutamic acid completely.

In the *L. casei* assay, the pteroylaspartic acid:pteroylglutamic acid inhibition index was about 1600, while in the *Escherichia coli* assay no inhibition was observed.

Another *L. casei* factor antagonist has been described by Martin, Tolman, and Moss (1947) and by Franklin *et al.* (1947). The compound was prepared by substantially the procedure of Waller *et al.* (1946, 1948) using 2,3-dibromobutyraldehyde instead of 2,3-dibromopropionaldehyde.

Martin and coworkers have called the active compound "7-methylfolic acid," but so far no data have been presented to substantiate this structure.

Franklin and associates, using the crude unpurified material as an antagonist for pteroylglutamic acid found "inhibition ratios" of 20:1 for *S. faecalis* R., 1000:1 for *L. casei*, and 3000:1 for the rat.

The effect of the antagonist could be reversed by increasing the concentration of pteroylglutamic acid.

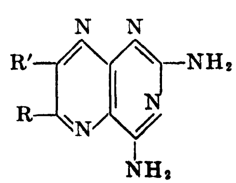
In rats, the pteroylglutamic acid deficiency syndrome was similar to but much more severe than that produced by succinylsulfathiazole, and oral lesions were observed that recall the oral lesions of monkeys deficient in Vitamin M (Day *et al.*, 1936).

Martin's group using their so-called "7-methyl *d*(-) folic acid" as an inhibitor for pteroylglutamic acid in the *S. faecalis* R. assay, reported an inhibition index of about 150, while in a later communication (Martin, Avakian, Tolman, *et al.*, 1947) they assign to 7-methylpteroyl-*l*(+)glutamic acid an inhibition index of 2. These workers also give inhibition indices for a number of other compounds: pteroylaspartic acid, 100; "7-methylpteroylaspartic acid," 100; "7-methylpteroic acid," 10; oxypteroic acid, inactive; oxypteroylglutamic acid, inactive; *N*-(4-[(4-quinazoline)amino] benzoyl)glutamic acid, inactive. This latter compound (Martin, Moss, and Avakian, 1947) showed some "folic acid" activity.

Still another pteroylglutamic acid inhibitor has been recently described by Seeger *et al.* (1947), namely *N*-[4-{[(2,4-diamino-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid. Using this pure material in the *S. faecalis* R. assay, the inhibition index was found to lie in the range of 1.9 to 0.4, depending upon the concentration of metabolite.

This pronounced inhibition of pteroylglutamic acid by its 4-amino analogue appears to be a function of the 4-amino group, since Daniel *et al.* (1947) have recently described experiments in which pteroylglutamic acid was inhibited by number of 4-aminopteridines, whereas the corresponding 2-amino-4-hydroxy, 2,4-dihydroxy, and 2-mercapto-4-hydroxy compounds showed little or no inhibitory effects.

The pteridines tested by Daniel and coworkers were prepared by Mallette *et al.* (1947) and by Cain *et al.* (1946). The inhibitory 4-amino-compounds had the following general structure.



The original papers should be consulted for a complete list of the pterins used in these experiments, but of all the compounds tested, 2,4-diamino-6,7-diphenylpyrimido(4,5-*b*)pyrazine and 2,4-diaminophenanthro(9,10-*e*)pyrimido(4,5-*b*)pyrazine were the most active inhibitors, using pteroylglutamic acid as the metabolite and *S. faecalis* R., *L. casei*, and *L. arabinosus* as the test organisms.

It is interesting to note that while both *S. faecalis* R. and *L. casei* require preformed pteroylglutamic acid and *L. arabinosus* is capable of synthesizing its own requirements of this substance yet competitive inhibition was observed with all three microorganisms.

It was also observed in the *L. arabinosus* assay, that 2 to 10 times as much pterin was required for growth inhibition if PABA was present.

In a later communication, Daniel *et al.* (1947) show that the growth of two other microorganisms which synthesize pteroylglutamic acid, *E. coli* and *Staphlococcus aureus,* is inhibited by these 2,4-diaminopterins. Furthermore a marked synergism was observed when low levels of the pterin and sulfathiazole were added to the culture medium.

The addition of pteroylglutamic acid completely overcame the growth inhibition caused by low levels of pterin and sulfathiazole and the effect of the compounds appeared to be competitive. If, however, the growth inhibitory effects of the pterin and the sulfathiazole were studied separately, it was found that the effect of adding pteroylglutamic acid was noncompetitive in nature.

Lampen and Jones (1946) have suggested that sulfonamides are antagonistic to pteroylglutamic acid by interfering with the biological synthesis of the pteroylglutamic acid, and Daniel *et al.* (1947) now suggest that the antagonistic effect of the 2,4-diaminopterins may be caused by interference with the use of pteroylglutamic acid and the above mentioned synergism is cited as evidence in favor of this hypothesis.

It seems desirable to point out that pure compounds should be used when studying the antagonistic or growth promoting action of these substances. In the preparation of pteroylglutamic acid and related compounds, the crude, unpurified reaction mixture may and in some cases has been shown to contain by-products with inhibitory properties. There is also evidence that two or more inhibitors having different biological action may occur in the same crude preparation.

XIV. Biological Activity

Pteroic acid, rhizopterin, and the various polyglutamic acid analogues of pteroylglutamic acid have varying activities for *Lactobacillus casei*, *Streptococcus faecalis* R., and the chick. If the biological activity of

pteroylglutamic acid is taken as 100, the various compounds exhibit the activities noted in Table I.

TABLE I

Biological Activities of Certain Pteroyl Compounds

Compound	Activity for			Reference
	L. casei %	*S. faecalis* R. %	Chick %	
Pteroic acid	No	Yes[1]	No	Angier *et al.* (1946)
Rhizopterin	No	10 × Molar activity	No	Rickes, Trenner, *et al.* (1947)
Pteroylglutamic acid	100	100	100	Angier *et al.* (1946)
Pteroyl-α-glutamylglutamic acid	0.5–1.0	0.5–1.0	Yes	Mowat, Hutchings, *et al.* (1948) Oleson, unpublished data
Pteroyl-α,γ-glutamyldiglutamic acid	0.5–1.0	0.5–1.0	Yes	Mowat, Hutchings, *et al.* (1948) Oleson, unpublished data
Pteroyl-γ-glutamylglutamic acid	Yes	Yes	Yes	Boothe, Mowat, *et al.* (1948) Oleson, unpublished data
Pteroyl-γ-glutamyl-γ-glutamylglutamic acid	Yes	2.0–4.0	Yes	Boothe, Mowat, *et al.* (1948) Oleson, unpublished data
Pteroylhexaglutamylglutamic acid	0.4	0.2	Yes	Pfiffner *et al.* (1945)

[1] Yes indicates the compounds are equimolecularly active.

Pteroic acid and rhizopterin are active for *S. faecalis* R. but not for *L. casei* or the chick. Almost the reverse is true when the biological potencies of pteroyl-γ-glutamyl-γ-glutamylglutamic acid are evaluated.

The two compounds with alpha linked peptides, pteroyl-α-glutamylglutamic acid and pteroyl-α,γ-glutamyldiglutamic acid, are essentially inactive microbiologically but are fully active for the chick. Whether all analogues containing alpha linked peptides will be microbiologically inactive is impossible to state at this time.

Vitamin B_c conjugate of Pfiffner *et al.* (1945) which has been designated as pteroylhexaglutamylglutamic acid (Pfiffner *et al.*, 1946) is inactive microbiologically but shows activity for the chick commensurate with its content of pteroylglutamic acid. After digestion with an enzyme

preparation from hog kidney or chicken pancreas, the hydrolyzed mixture has microbiological activities similar to those of pteroylglutamic acid.

The parallel microbiological inactivity of the conjugate and of the pteroyl compounds containing alpha linked peptides should not be construed as evidence that the conjugate has an α-peptide structure. There is no data in the literature indicating the configuration of the side chain of pteroylhexaglutamylglutamic acid.

From the microbiological and chick activities of the various compounds noted in Table I, it is most highly probable that the fractions designated variously as Factor U (Stokstad and Manning, 1938), Factor R (Schumacher *et al.*, 1940) and vitamins B_{10} and B_{11} (Briggs *et al.*, 1943) are, in fact, merely concentrates containing varying proportions of the above mentioned compounds. The fact that chicks can be raised on a diet containing known essentials is support for this view.

Scott *et al.* (1945) advanced the hypothesis that the lactone of either 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine or 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine facilitated the conversion of pteroyl-γ-glutamyl-γ-glutamylglutamic acid to pteroylglutamic acid which was then utilized by the chick. Hutchings, Oleson, and Stokstad (1946) under conditions of their experiments could not substantiate this finding. Jukes and Stokstad (1947) using diets similar to those of Scott and associates could show no beneficial effects on the addition of the lactone of 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine. The pteroyl-γ-glutamyl-γ-glutamylglutamic acid was as effective on a molar basis as was pteroylglutamic acid. It would seem, therefore, that the chick without dietary supplements can readily utilize the various conjugated forms of the vitamin.

XV. Vitamin B_C Conjugase (Pteroylglutamic Acid Conjugase)

The enzyme from hog kidney that is responsible for the degradation of pteroylhexaglutamylglutamic acid with the liberation of pteroylglutamic acid has been designated by Pfiffner *et al.*, (1946) as a carboxypeptidase. From the behavior of the enzyme obtained from various sources it appears that at least two distinct conjugases occur in nature. Bird *et al.* (1946) in a study of the distribution of the conjugate-splitting enzyme found that the pH optimum of the conjugase from animal organs was approximately pH 4.5 whereas the enzyme derived from turkey pancreas had a pH optimum of 7.0–7.5. Prior to this the enzyme present in chicken pancreas had been described as having a pH optimum of 7.0 (Laskowski *et al.*, 1945).

From the studies of Pfiffner and associates it was apparent that most natural materials contain a substance(s) that is inhibitory to the action

of the conjugase. In a continuance of this work Mims *et al.* (1947) demonstrated that nucleic acid either of the ribose or desoxyribose type was a strong *in vitro* inhibitor of the conjugase derived from hog kidney. Depolymerization of the nucleic acid by ribonuclease or thymonucleodepolymerase, respectively, or treatment with such reducing agents as cysteine, ascorbic acid, or hydrogen sulfide destroyed the inhibiting property of the nucleic acid. The pteroylglutamic acid conjugase from chicken pancreas was not inhibited by nucleic acid and the data was offered as further proof of the existence of at least two distinct conjugase enzymes.

Fragmentary data was presented by Mims and coworkers indicating that nucleic acid had an *in vivo* action by depressing the expected excretion of pteroylglutamic acid when normal subjects were fed specified amounts ot pteroylhexaglutamylglutamic acid.

An inhibition of the action of the pteroylglutamic acid conjugase from chicken pancreas and rat liver by the glutamic acid polypeptide of *p*-aminobenzoic acid (Ratner *et al.*, 1946) was reported by Sims and Totter (1947). The structural similarity of this compound and the *p*-aminobenzoic acid glutamic acid peptide moiety of pteroylhexaglutamylglutamic acid is obvious.

XVI. Quantitative Determination

The microbiological assay of pteroylglutamic acid has been adequately reviewed elsewhere (Stokstad and Hutchings, 1947).

For the chemical determination ot the pteroyl compounds, Hutchings, Stokstad, Boothe, *et al.* (1947) have advanced a method based on the reductive cleavage of this group of compounds into their pteridin and aromatic amine components. The difference between the aromatic amine before and after reduction as determined by the method of Bratton and Marshall (1939) is used as a measure of the pteroyl derivative present.

The reduction is satisfactorily carried out in 0.5 *N* hydrochloric acid with zinc dust. A small amount of gelatin (0.005%) must be included in the reducing menstruum to inhibit the reductive destruction of the liberated amine. Under these conditions the reduction of pteroylglutamic acid proceeded essentially to completion.

The method is satisfactory for the determination of pteroylglutamic acid, its analogues and derivatives and for concentrates from natural sources that contain the active compound in excess of 5%.

The compounds in natural materials, both inorganic and organic, that may interfere with the determination have not been assessed. Additional work should be forthcoming along these lines.

XVII. SUMMARY

Degradation and synthesis have established the structure of the naturally occurring compounds variously designated as Vitamin B_c, liver *L. casei* factor, the fermentation *L. casei* factor, and rhizopterin. All of these compounds have a similar nucleus consisting of 4-[(2-amino-4-hydroxy-6-pteridyl)methyl] aminobenzoic acid. This compound or its formyl derivative (rhizopterin) exhibits activity for *S. faecalis* R., but is inactive for *L. casei*, the chick, and the rat. Pteroylglutamic acid, *N*-[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]-amino}-benzoyl]-glutamic acid, (Vitamin B_c) (liver *L. casei* factor) is the simplest structure that shows both microbiological and animal activity. Extension of the glutamic acid side chain in either the α- or γ-position alters the microbiological activity, but the compounds retain equimolecular activity for the chick.

Certain compounds structurally related to pteroylglutamic acid have been prepared and shown to have antipteroylglutamic acid activity.

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Vitamin K

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CONTENTS

I. Introduction

Following the realization of the clinical significance of vitamin K and the isolation of the vitamin, a number of reviews were published describing the fundamental investigations in this field. (Almquist, 1941; Brinkhous, 1940; Butt and Snell, 1941; Dam, 1942; Dam and Glavind, 1940; Doisy *et al.*, 1941; Koller, 1941; Riegel, 1940.) The present article will deal with the development which has taken place since then.

It must be remarked that when the author wrote a review on Vitamin K several years ago (Dam, 1942), it was possible to present a rather well-rounded picture of all the fundamental facts then available. The information which has been gathered in recent years is much less coherent and comprises several observations (real or postulated) that have been discussed in the literature, without definite conclusion. It would be impossible to mention all the contributions which have appeared, and this review is, therefore, limited to what the author thinks will give a fair representation of the development.

II. Vitamin K Active Compounds

Some new substances have been added to the list of vitamin K active compounds[1] during recent years, some of these are: 1-acetoxy-2-methyl-4-naphthyl sodium phosphate and the corresponding sulfate (Baker and Carlson, 1942; Baker *et al.*, 1942). The compound which is formed by boiling menadione with sodium hydrogen bisulfite (Moore, 1941) has been identified as menadione bisulfite (*J. Am. Med. Assoc.*, 1943). E. Chu (Chu, 1945) states that 4-(3′-methyl-4′-hydroxynaphthylazo)-benzenesulfonamide is almost as potent as menadione,[2] and 5-methyl-4,7-

[1] Most of the previously known vitamin K active compounds are listed by earlier reviewers, such as Dam (1942) or Rosenberg (1942).

[2] The committee on nomenclature of the American Medical Association in 1941 (*J. Am. Med. Assoc.*, 1942) adopted the term "menadione" and authorized its use as a nonproprietary name to describe the substance 2-methyl-1,4-naphthoquinone. The U.S.P. used this term for the first time in its XIIth edition. The two naturally

thionaphthenequinone, an isostere of menadione, has been found to have about 3% of the vitamin K activity of menadione (Tarbell *et al.*, 1945). This latter finding is rather peculiar since it might be expected that this compound might antagonize vitamin K.

Mikhlin (Mikhlin, 1942; 1943) has reported that corn stigma contains a substance, termed by him vitamin K_3, which accelerates blood coagulation in normal animals and in patients. It is said to be alcohol-soluble and water insoluble; however, its differentiation from vitamin K_1 is not clear. Other workers have used the term K_3 to designate menadione.

Mentzer and Meunier (Mentzer and Meunier, 1943; 1944) claimed that 3-methylchromone and 2-carboxy-3-methylchromone had a considerable vitamin K activity. Glavind (1948) could not substantiate this claim.

Vitamin K_2 previously isolated from putrified proteins has been found in cultures of a spore-forming soil bacillus, *Bacillus brevis* (Tishler and Sampson (1948)).

III. Methods for the Determination of Vitamin K and Related Substances

1. Blood Clotting Tests

The chick assay has been reexamined by Quick and Stefanini (1948) and a test with rats fed a diet containing sulfadiazine has been proposed by Kornberg *et al.* (1944a, 1944b) (cf. VI, 7).

2. Chemical Tests

Chemical tests have received particular attention. Several reactions, more or less specific for quinones of the vitamin K type, have been proposed as assay methods, but methods for extracting small, i.e., physiological, quantities of vitamin K or its substitutes from biological material do not seem to have been sufficiently worked out.

The following reactions have been proposed:

A. Colorimetric Methods

Reactions with Dinitrophenylhydrazine (Novelli, 1941; Novelli and Conticello, 1944; Menotti, 1943; Bosecke and Laves, 1943; Vonesch, 1941, 1942a, 1942b, 1942c, 1943; Tastaldi *et al.*, 1945; Giral and Iglesias, 1942; Armstrong, 1943, 1944). The modification by Menotti will be described briefly.

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occurring K vitamins: K_1 and K_2 are 3-phytylmenadione and 3-difarnesylmenadione respectively.

To an alcoholic solution of menadione is added a solution of dinitrophenylhydrazine in aqueous hydrochloric acid. By heating to 70°C. for 15 minutes a hydrazone is formed. After cooling to room temperature an alcoholic solution of ammonia is added and the mixture diluted with alcohol to a fixed volume. The reaction results in a blue color which, together with the yellow color of the reagent, makes the solution appear green. The maximum absorption is at 6350 A. For the details see the publication by Menotti (1943).

The color develops with methylnaphthoquinone but not with vitamin K_1. The method of Menotti is said to be reliable for the determination of 2-methyl-1,4-naphthoquinone in quantities as low as 0.05 mg. The color is very stable.

Craven's Reaction (1931) with Ethyl Cyanoacetate. This has been used by Pinder and Singer (1940) and Kofler (1945) for the determination of methylnaphthoquinone with which it gives a blue color; no color is obtained with vitamin K_1. The method is less sensitive than the reaction with dinitrophenylhydrazine. Fieser (1941) has briefly examined the reaction.

The Reactions with Sodium Diethyl Dithiocarbamate (Irreverre and Sullivan, 1941). With vitamin K_1 a blue color develops while methylnaphthoquinone gives a pink color. The blue color is not stable, it reaches a maximum 7 minutes after the reagents have been mixed, whereafter it fades. The sensitivity is said to be about 5γ/ml.[2a]

Reactions with Sodium Ethylate (Dam *et al.*, 1939; Karrer, 1939; Fieser, *et al.*, 1939). Vitamin K_1 and vitamin K_2 both give a blue color which later changes to red-brown. Methylnaphthoquinone gives a green color which also changes to red-brown. A variation of this method, using sodium methylate instead of ethylate, has been used by Almquist and Klose (1939). Fernholz *et al.* (1939), used the reaction with sodium methylate and suggested that there might be some vitamin K active substance in plant material which does not give the color reaction. Kofler (1946) states the opposite, viz., that plant material contains another quinone which gives a strong blue color with sodium ethylate and has a very low activity, if any at all, as vitamin K. Bosecke and Laves (1943) have used the method for the determination of methylnaphthoquinones using the Pulfrich *Stufenphotometer*, filters S 53 and S 50, and 2 hours reaction time. The method is less sensitive than the reactions with dinitrophenylhydrazine and diethyl dithiocarbamate.

Carrara *et al.* (1940; 1941) have used an alcoholic solution of sodium hydroxyde instead of sodium ethylate. Colucci (1946) has modified the reaction so that it can be used for methylnaphthoquinone or methylnaphthohydroquinone diacetate in oil solution.

[2a] γ = micrograms.

Other Color Reactions. Methylnaphthoquinone reacts with 2,6-dichlorophenolindophenol and sodium hydroxide yielding first a brown solution which turns green after shaking. The green color can be taken up in amyl alcohol (Bosecke and Laves, 1943).

Martinson and Meerovich (1945) and Meerovich (1946a; 1946b) report that methylnaphthoquinone gives a red color on addition of aniline. He used this color for quantitative determination (the reaction has also been described by Fieser, 1941).

Berlin (1940) indicates a procedure whereby methylnaphthohydroquinone diacetate in methylacetamide gives a red color on addition of sodium hydroxide. He records the time from the addition of sodium hydroxide to the appearance of the red color. The recorded time is inversely proportional to the concentration of the methylnaphthohydroquinone diacetate. If methylnaphthoquinone or methylnaphthohydroquinone is used instead of the diacetate the color appears almost instantaneously. The method can be used for the determination of methylnaphthoquinone in pharmaceutical preparations. The smallest amount determined seems to be 750γ/ml.

Greco and Argenziano (1946) propose a reaction with phenyl hydrazine whereby an azo derivative not a phenyl hydrazone is obtained. This derivative, dissolved in sulfuric or phosphoric acid, gives a red-violet color that can be measured in the colorimeter. The method seems to be useful for vitamin K_1 and methylnaphthoquinone.

Schulek and Rózsa (1941a; 1941b; 1941c; 1941d; 1942) have observed a color reaction between methylnaphthoquinones and strong acids (concentrated phosphoric acid, 50% sulfuric acid, 20–38% hydrochloric acid). Heating on a water bath gives a red color. The red substance separates on cooling and can be taken up again in various solvents. The method is not very sensitive.

Scudi and Buhs (1941) have introduced a rapid colorimetric method for the determination of menadione. The method consists in the development of a yellow color when cysteine and sodium hydroxide are added to a solution of menadione. The solution may be alcoholic, aqueous or ether-alcoholic, or blood plasma. The method seems also to be applicable to urine and seems to be useful for concentrations as low as 1 or 2γ/ml. It can also be used for aqueous solutions of 2-methyl-1, 4-naphthoquinone-3-sulfonate.

Menotti (1942) has indicated a colorimetric method for the determination of 4-amino-2-methyl-1-naphthol, a vitamin K active pharmaceutical preparation which does not occur in nature. This method is based on the reaction between sodium pentacyanoamineferroate and the before mentioned compound in alcohol solution, whereby a blue color is obtained.

The sensitivity is said to be low, 0.5γ. Methylnaphthoquinone gives no color.

Fieser (1941) has examined several color reactions for methylnaphthoquinone and found that only two of them can be used also for vitamin K_1. These two reactions are those with ethyl cyanoacetate (Craven, 1931) and ethanolamine. Other substances which according to Fieser react with methylnaphthoquinone are: ethyl acetoacetate, pyruvic acid, aniline (cf. Meerovich 1946a, 1946b) ethanolamine, lysine, thioglycolic acid, and cysteine (cf. Scudi and Buhs, 1942b).

B. REDOX METHODS

Pinder and Singer (1940) have proposed to determine methylnaphthoquinone by titration with titanous chloride using potassium indigo bisulfonate as indicator.

Trenner and Bacher (1941) have proposed another redox method for vitamin K_1 and methylnaphthoquinone. This method consists of two stages; viz., the catalytic reduction of the quinone to hydroquinone and reoxidation of an aliquot of the latter with 2,6-dichlorophenolindophenol. They indicate that the highest accuracy (about 1%) is obtained with amounts of K_1 of about 500 to 1000γ/ml. Using suitable microglass equipment it should be possible to work with as little as 20γ/ml. The method requires special glass equipment which makes it possible to carry out the catalytic reduction, and the titration with dichlorophenolindophenol without exposing the substance to air.

According to Schulek and Rózsa (1941c; 1941d; 1942) methylnaphthoquinone can be determined by cerimetric titration. The quinone is first reduced to hydroquinone with a solution of stannous chloride in hydrochloric acid, the hydroquinone is extracted with chloroform and titrated with a cerium sulfate solution. *p*-Ethoxychrysoidine serves as indicator. Another redox method has been described by Rosin *et al.* (1941).

C. COLORIMETRIC REDOX METHODS

Scudi and Buhs (1941; 1942a) and Scudi (1941) have proposed a colorimetric redox method based on the principle used by Trenner and Bacher (1941). The method can be used for vitamin K_1 and for methylnaphthoquinone. The quinone dissolved in butanol is reduced catalytically in the presence of phenosaphranine. To the hydroquinone thereby formed is added an excess of 2,6-dichlorophenolindophenol in butanol under the exclusion of oxygen. The decreasing color intensity is then a measure for the amount of quinone originally present. The method can be used for solutions containing as little as 5γ K_1/ml. It

requires the same special glass apparatus as the method of Trenner and Bacher.

D. POLAROGRAPHY

Hershberg *et al.* (1940) state that they can determine vitamin K_1 by means of the polarograph in concentrations as low as 50γ in a solution of 2.5 ml. acetone containing 10 mg. LiCl.

E. FLUOROMETRY

According to Kofler (1945) methylnaphthoquinone can be determined fluorometrically, since it forms a fluorescent compound by condensation with *o*-phenylenediamine. The blue fluorescence of an alcoholic solution of this compound is so intense that it can be detected in solutions down to 0.1γ in 5 ml. alcohol.

F. SPECTROSCOPIC METHOD

The spectroscopic method has been given renewed attention by Ewing *et al.* (1943).

The following two publications on the determination of vitamin K were not available to the reviewer before the article was finished: Zakharova and Devyatnin (1944) and Schoen (1945).

IV. Mode of Action of Vitamin K in Prothrombin Formation

The way in which vitamin K contributes to the formation of prothrombin has been considered largely from the speculative point of view.

Based on the earlier papers of Baumberger (1941), and Bernheim and Bernheim (1940), in which a possible role of thiol groups in fibrin formation was discussed, Lyons (1945) formulated a definite scheme for the mode of action of vitamin K. According to this scheme vitamin K is a functional part of the thrombin molecule and the clotting of fibrinogen occurs in two stages: 1) the liberation of blocked thiol groups in fibrinogen brought about by one component of thrombin, and 2) an oxidation, probably by a naphthoquinone complex in thrombin, converting protein —SH into protein —S—S— protein (fibrin). Lyons claims to have demonstrated the presence of a naphthoquinone derivative in thrombin by means of Novelli's (1941) reaction and Craven's ethyl cyanoacetate test (1931).

Attempts to confirm this latter finding in the reviewer's laboratory have turned out negatively.

Chargaff and Bendich (1943) have shown that a fibrinogen solution may clot on addition of several substances such as chloramine T, potassium 1,4-naphthoquinone-2-sulfonate, sodium 1,2-naphthoquinone-4-sulfonate, ninhydrine, alloxan, and salicylaldehyde while two sulfonic acids

of 2-methyl-1,4-naphthoquinone were inactive. They do not claim, however, that these observations explain the mode of action of vitamin K.

The relation of vitamin K to the two components of prothrombin, whose existence was maintained by A. J. Quick (1943) (cf. the criticism by Seegers *et al.* (1945)), should be investigated as well as its relation to the factor V in blood coagulation described by Owren (1947) and the factor in plasma which accelerates the activation of prothrombin described by Ware *et al.* (1947). Preliminary steps in this direction have been made by Quick (1943), by Dam (1948), and by Dan and Søndergaard (1948).

The question whether large doses of vitamin K can raise the prothrombin level above normal has been dealt with by Field and Link (1944). They report that the oral administration of large doses of menadione (5 to 20 mg./kg.) to dogs, rabbits, and rats result in hyperprothrombinemia persisting for several days depending on the dose.

Quick (1946) denied that large doses of menadione, synkayvite etc. produce hyperprothrombinemia.

Field *et al.* (1944) also reported that hyperprothrombinemia could be induced by methylxanthine.

An important problem is whether vitamin K and its substitutes are converted into menadione in the body, in which case the latter substance may be looked upon as the true prothrombinogenic factor. Richert (1944) attacked this problem by testing the urine of fowls and rabbits for menadione after the animals had been given certain menadione derivatives, orally or parenterally, in rather large doses. The substances tested were: 4-amino-2-methyl-1-naphthol, 2-methyl-1-tetralone, 2-methyl-1,4-naphthohydroquinone diphosphate. Craven's ethyl cyanoacetate color test was used for the determination of menadione in the urine. A smaller or larger fraction of all the compounds listed was recovered in the urine as menadione.

Meerovich (1946a; 1946b) found that when the bisulfite derivative of 2-methyl-1,4-naphthoquinone, "Vitanol," was injected into the vena jugularis of rabbits some menadione could be found in the urine. Meerovich used the color reaction with cysteine and alkali (Scudi and Buhs, 1942b; Fieser, 1941) for the determination of menadione plus the bisulfite compound, and the reaction with aniline for the determination of menadione.

Whether vitamin K_1 and K_2 are also converted into menadione in the body does not seem to have been definitely settled.

The assertion by some authors (Shemiakin *et al.*, 1943) that phthalic acid should be the intermediary compound which carries the prothrombinogenic effect has been refuted by Dam (1942), Karrer and Koller (1943), and Blumberg and Arnold (1944).

V. Effects of Vitamin K and Related Substances on Other Processes Other than Prothrombin Formation

1. Possible Influence on Bleeding Tendency in Other Ways than by Prothrombin Formation

An interesting observation on factors which may influence the occurrence of hemorrhages in vitamin K deficient animals has been made by Brown *et al.* (1947). They reared female rats to maturity on an artificial diet deficient in vitamin K. If lard was removed from the diet and the females allowed to bear litters, brain hemorrhages occurred in the offspring. If the diet contained either lard or vitamin K the hemorrhages did not occur. They do not ascribe this observation to vitamin K occurring in the lard but suggest that some substance which acts normally to maintain capillary strength is not synthesized by the body when the diet is low in fat and vitamin K is absent. The coagulation time of the whole blood of females that produced hemorrhagic offspring and of young with visible hemorrhages was said to be normal.

A possible influence of vitamin K on serum antithrombin is mentioned in section VII, 1.

2. Postulated Influence on Blood Pressure

Oppenheimer *et al.* (1944) report that certain quinones, including menadione, have more or less antipressor properties when tested in hypertensive animals. They do not consider this effect specific for vitamin K, however, and state that in the case of menadione rather large doses must be given. Findings similar to these have been published independently by Schwarz and Ziegler (1944).

Moss and Wakerlin (1946) did not find any effect of vitamin K in daily doses of 60 mg. for 6 months in experiments with dogs rendered hypertensive by the Goldblatt technique. A further negative report has been published by Ferregra (1944).

3. Postulated Effect on Proteolytic Enzymes

According to Harkevitch (1942) vitamin K exerts a definite activating action upon trypsin.

4. Postulated Lipotropic Effect

Honorato and García Merino (1942–43) found fatty degeneration of the liver in vitamin K deficient chicks and attributed lipotropic properties to menadione analogous to the properties of choline. A reinvestigation by Field and Dam (1945) failed to confirm this assertion.

5. Influence on Metabolic Processes and Growth of Heterotrophic Organisms

Summerson (1943) examined the effect of menadione on the metabolism of isolated animal tissue. He found that in the presence of this substance in a concentration of approximately 10^{-4} *M*, rabbit exudate leucocytes respiring in Ringer's bicarbonate-glucose medium show an inhibition of aerobic lactic acid formation of 50% or more; at the same time there was a 10–15% increase in the rate of oxidative metabolism and a slight rise in R.Q. Rabbit bone marrow slices were found to show the same general effect. Rat liver slices also showed increased oxygen uptake while the utilization of lactate diminished. There was no change in R.Q. Under anaerobic conditions the conversion of added glucose to lactic acid by leucocytes was completely inhibited by menadione but the endogenous glycolysis of leucocytes and liver was not affected. The effect of menadione was found to fall off with time, perhaps due to inactivation by combination with cell proteins, presumably their SH groups, since the yellow protein-menadione complex obtained from leucocytes was found to be spectrophotometrically similar to the yellow compound formed by the interaction of menadione and cystein.

Scudi (1942) found that menadione but not vitamin K_1 causes formation of methemoglobin when added to whole blood.

Studies on the inhibitory effect of quinones, including menadione, on lactic acid formation by bacteria and bacteriostatic effect of such quinones have been published by Armstrong and Knutson (1943) and Armstrong *et al.* (1943). These studies are dealt with in this review in the section treating the postulated effect of vitamin K-like substances on dental caries.

According to Atkins and Ward (1945) Gram-negative organisms and a few streptococci are less susceptible to the antibacterial effects of vitamin K analogues than the majority of Gram-positive organisms.

Gonzalez (1945) compared the antibiotic effect of certain quinones on *Penicillium notatum* and found menadione more effective than benzoquinone and hydroquinone. The mechansim of the antibacterial action of quinones and hydroquinones were studied by Geiger (1946). Naphthoquinones as antagonists of the anti-streptococcal action of iodinin (di-*N*-oxide of a dihydroxyphenazine) were studied by McIlwain (1943).

2-Methyl-4-amino-1-naphthol hydrochloride, designated vitamin K_5 by some workers, has been found to possess antibiotic properties towards various fungi and to check fermentation of glucose by yeast (Pratt *et al.*, 1948). The effect is ascribed to altered redox potentials. Practical application of this observation has been suggested.

Ball *et al.* (1947) examined the effect of a number of 2-hydroxy-3-

alkylnaphthoquinones on erythrocytes, liver slices, and yeast cells and found them to be very potental inhibitors of respiratory processes, acting below cytochrome c and above cytochrome b in the main chain of respiratory enzymes. These substances, which include phthiocol, are much less active than vitamin K or than menadione and it cannot be said at present whether these findings have any relation to vitamin K.

Schmidt and Büsing (1942) have published a report according to which a principal source of vitamin K (viz., *Escherichia coli*) grows slowly in the intestine of chicks fed a vitamin K-free diet, and that vitamin K restores the coli flora. This report awaits further confirmation.

It has previously been reported that the growth of *Mycobacterium paratuberculosis* (Johne's bacillus) is greatly stimulated by naphthoquinone derivatives of vitamin K type (Woolley and McCarter, 1940). In an investigation on factors influencing the growth of Johne's bacillus Glavind and Dam (1948) could not find any growth stimulating effect of such naphthoquinones and concluded that the growth stimulating factor which is present in tuberculin, for instance, must be different from vitamin K. The strains of Johne's bacillus used by the two groups of workers were not the same.

6. Influence on Photosynthesis, Respiration, etc. in Photosynthesizing Microorganisms

The role of vitamin K in photosynthesis has received some attention. Dam (1944) found vitamin K in the green alga *Chlorella vulgaris* and also in some photosynthesizing bacteria, although the latter contained less than *Chlorella*. Menadione, added as powder to photosynthesizing *Chlorella*, stopped photosynthesis after having remained in contact with the organism for several hours.

Gaffron (1945) working with the algae *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, and *Scenedesmus sp.* D_3, tested under varied conditions the effects of menadione and phthiocol as well as water-soluble esters of menadione on photosynthesis, respiration, photoreduction, and oxyhydrogen reaction. He found that there is hardly a metabolic process in algae which cannot be influenced by small amounts of the two vitamin K-like substances. Small concentrations of the two naphthoquinones stimulated respiration, while large concentrations inhibited it. The water-soluble esters had less effect.

With very small doses of the two naphthoquinones it was possible to stimulate respiration without inhibiting photosynthesis but otherwise these compounds (not the water-soluble esters) were found to be very powerful inhibitors of photosynthesis. When the naphthoquinones are used in still higher concentrations (e.g., menadione as powder) they

damage the cells progressively, and respiration and photosynthesis are stopped.

Gaffron classifies the two methylnaphthoquinones as poisons in line with hydroxylamine and *o*-phenanthroline and thinks that they may form menadione compounds with heavy metal catalysts. On the other hand their stimulating effect on respiration and capacity to transfer oxygen differ from those of hydroxylamine and *o*-phenanthroline. They may act as oxidoreduction catalysts and may serve in more than one way in the metabolism of the plant.

7. *Effect on Mitosis*

F. E. Lehmann *et al.* (1942; 1945) have shown that menadione inhibits mitosis in *Tubifex* eggs. Mitchell and Reuss (1947) experimented with tissue cultures of fibroblasts from the choroid and sclerotic tissues of the chick embryo eye as follows:

Group A: cultures were started with synkayvite (tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid) in the medium so as to give a concentration of 3×10^{-6} *M*.

Group B: cultures were started in the same way but after 18 hours an x-ray irradiation of 244 röntgen was given.

Group C: cultures started without synkayvite and irradiated as B after 18 hours.

Counts of cell division were made 24 hours, 36 hours, and 48 hours after the cultures were started. Group B (synkayvite + x-ray) showed a very considerable reduction (about 90%) of cell division, whereas the cell division was much more in the two other groups.

In another series of experiments the concentration of synkayvite was varied without combination with x-ray treatment. It was found that the mitotic counts declined with increasing concentrations of synkayvite.

Experiments with human patients were tried and were found not inconsistent with the tissue culture experiments although at the present stage of investigation no therapeutic value was claimed.

VI. Substances Causing Hypoprothrombinemia and Their Relation to Vitamin K

1. *Dicumarol*

Substances that cause a lowering of prothrombin, which can be compensated more or less by large doses of vitamin K, have been studied by several authors. One of the most discussed substances is *dicumarol* (3,3′-methylene-bis(4-hydroxycoumarin)). The prothrombin-lowering effect of dicumarol was known (Roderick, 1931) before the substance was isolated (Campbell and Link, 1941). After the isolation and identification of dicumarol some authors, such as J. Lehmann (1942), assumed that

dicumarol acts by a competitive interference with the action of vitamin K; that is, because of a certain structural resemblance to vitamin K, dicumarol should replace vitamin K in some enzyme system involved in the formation of prothrombin. A direct proof for this hypothesis does not seem to have been presented. On the contrary, there are experiments which make this type of explanation seem less likely. Jansen and Jensen (1942) examined a number of compounds representing variations of the dicumarol molecule. In addition to dicumarol they found only 3,3′-ethylidene-bis(4-hydroxycoumarin) active whereas a substance such as 3-methyl-4-hydroxycoumarin, which resembles vitamin K much more closely was inactive. They therefore believe that the effect of dicumarol is not due to a structural resemblance to vitamin K. Glavind and Jansen (1944) determined the amount of dicumarol necessary to overcome the effect of varying doses of simultaneously administered vitamin K (in the form of 2-methyl-1,4-naphthohydroquinone disulfate). They found that the necessary dose of dicumarol increased only from 0.03 to 0.2 (that is, about 7 times) when the amount of vitamin K was increased from 0.00001 to 0.025 (that is, 2500 times). (Both compounds are expressed in terms of milligrams per gram of body weight of the rabbit.) They conclude that this result does not support the replacement theory, since it would be expected that the amount of dicumarol required to replace vitamin K would increase very much (according to the law of mass action) when the dose of the vitamin K compound is increased more than 2000 times. Woolley (1947), aware of Jansen and Glavind's paper, nevertheless seems to favor the replacement theory.

Eriksen *et al.* (1945) found that 3,3′-methylene-bis(3,4-dihydro-4-hydroxycoumarin ("hydrodicumarol") exerts an action similar to that of dicumarol and suggest that dicumarol is partly converted into hydrodicumarol in the body. They studied the mechanism of the anticoagulant effect of hydrodicumarol and found it to be of a rather complex nature.

Meunier *et al.* (1943) report that methylene-bis(hydroxy-1,4-naphthoquinone) (derived from phthiocol) antagonizes the effect of vitamin K, indicating that the "doubling" of the molecule produces an antagonist.[3] This fact might be interpreted in favor of the replacement theory.

[3] The words "antagonize" and "antagonist" in this connection are often taken to indicate antagonism due to replacement, but it is not always clear whether an author wishes to have the words so understood. Logically the terms do not necessarily implicate any specific mechanism by which the antagonistic effect is brought about. In cases where the effect of the antagonist is easily overcome by a suitable increase of the dose of vitamin K, and vice versa it is likely that the replacement theory holds, provided that beginning liver damage, or blocking of supply of vitamin K by intestinal bacteria, or interference with absorption from the intestine do not occur.

Meunier and Mentzer (1942) found that the effect of dicumarol is antagonized by 2-methyl-4-hydroxycoumarin, the substance which Jansen and Jensen found did not antagonize vitamin K.

It is worthwhile to remember that dicumarol exerts a toxic effect which manifests itself in other ways than by hypoprothrombinemia; it causes loss of weight and the animals often die without bleeding. Emmel and Dam (1944) did not find any indication of fatty degeneration in the liver of dicumarol-poisoned chicks, but the possibility should be considered that the general intoxication might interfere with prothrombin formation just as it is seen in the initial stages of chloroform poisoning where it is also possible to partially compensate the hypoprothrombinemia by ingestion of vitamin K.

Jansen (1944) believes in the intoxication theory, and reports liver damage in dicumarol poisoned rabbits in accordance with the observations of Wakim *et al.* (1943). The intoxication theory is further supported by the observations on ducklings made by Rigdon and Varnadoe (1947) according to which there seems to be a relationship between the toxicity of dicumarol and the amount of vitamin A in the diet.

Quick and Stefanini (1948) report that vitamin K_1 and menadione are about equally effective in counteracting the hypoprothrombinemic effect of dicumarol.

2. Isosteres of Vitamin K with Antagonistic Action

A rather interesting example of antagonism, viz., that of α-tocopherolquinone, vitamin K, and vitamin E, was studied by Woolley (1945). He found that α-tocopherolquinone opposes the effect of vitamin E and that this antagonistic effect can be abolished by vitamin K but not by vitamin E. Another antagonist to vitamin K is, according to Woolley, 2,3-dichloro-1,4-naphthoquinone.

Smith *et al.* (1946; 1947) studied the antagonistic effect of derivatives of phthiocol with substituents in the methyl group. The hemorrhagic effect of their compounds was noted during the examination of the toxicity of various antimalarial drugs. They found hemorrhagic and toxic effects of such compounds as 2-(3-cyclohexylpropyl)-3-hydroxy-1,4-naphthoquinone (called S 5090), 2-(2-methyloctyl)-3-hydroxy-1,4-naphthoquinone and 2-(3-(decahydro-2-naphthyl)propyl)-3-hydroxy-1,4-naphthoquinone. The effects were antagonized partially by menadione and completely by vitamin K_1. The compounds produced only minimal changes in the liver, caused no destruction of prothrombin *in vitro* and possessed only slight bacteriostatic activity against *Escherichia coli*, the principal source of vitamin K in the intestinal tract. They conclude that these compounds exert their antagonistic effect by competition with

vitamin K. It is noteworthy that they found vitamin K_1 to be more effective in compensating the hemorrhagic effect than menadione.

3. Salicylates

The production of hypoprothrombinemia by the ingestion of salicylates by rats maintained on a ration low in vitamin K, was reported by Link *et al.* (1943). They found that vitamin K in the form of menadione protects the rats against the hypoprothrombinemia induced by salicylic acid and they considered the possibility that degradation of dicumarol to salicylate in the body might explain the hemorrhagic effect of dicumarol. A similar effect of salicylate (and acetylsalicylate) in human patients was noticed by Meyer and Howard (1943), though the increases of prothrombin time and coagulation time were rather small, and by Rapoport *et al.* (1943) and Shapiro *et al.* (1943). According to the latter authors acetylsalicylic acid appears to be a more potent agent than sodium salicylate. Lester (1944) reported that no salicylate appeared in the urine after ingestion of dicumarol by rats. Since the effect of salicylate is much less than the effect of dicumarol it seems unlikely that dicumarol acts via salicylic acid. Jacques and Lepp (1947) report that sodium salicylate changes the prothrombin only when given orally. They suggest that salicylate may be converted to dicumarol or a substance with similar prothrombinopenic properties by bacterial action in the intestinal tract. The use of vitamin K in salicylate therapy is still being considered. Quick (1945) doubted that salicylates produce dangerously lowered prothrombin and suggested that salicylates may cause hemorrhages in other ways, perhaps through thrombocytopenia.

4. Dihydroxystearic Acid

Lockhart *et al.* (1942) found that the feeding of 25% synthetic fat containing dihydroxystearic acid (1 molecule of this acid +2 molecules of other fatty acids in the triglyceride) in vitamin K-deficient rat diets resulted in rapid development of vitamin K deficiency symptoms. Small amounts of menadione prevented the symptoms. This interesting observation was further investigated by Nightingale *et al.* (1947). They found that dihydroxystearic acid blocks the synthesis of vitamin K by intestinal bacteria without killing the bacteria. Thus their observations deal with a case where a substance definitely aids the production of K deficiency without simply acting as a "competitive antagonist" for the vitamin.

5. Vitamin A in Massive Doses

The production of hemorrhages by feeding excessive amounts of vitamin A in the form of either crystalline vitamin A or halibut liver oil

was noted by Rodahl and Moore (1943), Moore and Wang (1945) and by Light *et al.* (1944). The latter authors, as well as Walker *et al.* (1947), found that the condition is associated with hypoprothrombinemia and may be prevented by the simultaneous addition of vitamin K in relatively small amounts.

Quick and Stefanini (1948) confirmed this observation in rats but could not find a similar effect when chicks were used as experimental animals. They point out the possibility that the effect is due to interference with bacterial synthesis of vitamin K.

6. *Mineral Oil*

Production of vitamin K deficiency in rats by ingestion of large amounts of mineral oil, whereby the intestinal absorption of the vitamin is reduced, was reported in 1940 by Elliott *et al.* and later by Javert and Macri (1941). The paper of the first mentioned authors contained the statement that in addition to vitamin K, activated ergosterol definitely improved the deficiency. This statement does not seem to have attracted sufficient attention.

7. *Sulfa Drugs*

Kornberg *et al.* (1944a; 1944b) have recommended the use of sulfadiazine in producing vitamin K deficiency in rats for the purpose of biological determination of vitamin K. The sulfadiazine is supposed to act by suppressing the intestinal flora. Granados and Dam (1945) also reared rats on similar diets. While the rats developed vitamin K deficiency they showed severe signs of sulfa-drug poisoning (loss of weight, weakness, hematuria, urinary calculi, and early death) and were scarcely of any value as test animals.

Seeler *et al.* (1944) and Mushett and Seeler (1947) studied the hypoprothrombinemia resulting from the administration of sulfaquinoxaline (2-sulfanil-amido quinoxaline). They found that the hypoprothrombinemic action cannot be attributed solely to either the sulfamido or the quinoxaline portion of the molecule, but is due to the combination of both. They report that vitamin K_1 is many times as potent as menadione in compensating the effect of sulfa quinoxaline.

8. *Other Compounds*

Procaine used for spinal anesthesia is said to minimize the clotting power of the blood while vitamin K counteracts this effect (Levy and Conroy, 1947).

VII. Application of Vitamin K in Human Medicine

1. *The Well Established Therapeutic Applications of Vitamin K in Cases with Generally Recognized Hypoprothrombinemia*

The well established therapeutical applications of vitamin K against bleeding tendencies in surgical patients with obstructive jaundice, against bleeding tendencies in the newborn, and in celiac disease etc., has been so well described before that it does not seem necessary to go further into details in this review. The following supplementary information may suffice.

Marx and Dyckerhoff (1943) reported that in obstructive jaundice the coagulation anomaly is caused, not only by lack of prothrombin, but also by increased antithrombin. The increase in antithrombin is due to the increase in the affinity of one or more blood proteins for thrombin. Vitamin K is said to influence advantageously not only the prothrombin but also the abnormal condition of the blood proteins.

The exact daily requirement of human adults for vitamin K has not yet been determined, but figures for the newborn are available. Sells *et al.* (1941) reported that the requirement during the first few days after birth is extremely low (1–2 γ of 2-methyl-1-amino-4-naphthol hydrochloride). This claim has largely been substantiated by other investigators. Thus, Larsen (1943) found that 1 γ of 2-methyl-1,4-naphthohydroquinone disuccinate is sufficient for the newborn when given intravenously every day, and that 5 γ of this substance or of pure vitamin K_1 is sufficient when given orally daily.

Sarah H. Hardwicke (1944) working with the tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid, "Synkayvite," found the minimal effective daily dose to lie somewhere between 5 and 0.5 γ. If it is intended to produce and maintain normal prothrombin through the first 7–10 days after birth by one single dose, the amount given should be higher.

The cause of the hypoprothrombinemia of the newborn was studied from different points of view (Dam *et al.*, 1942; Glavind *et al.*, 1942; Plum and Uldall, 1942; and Venndt and Plum, 1942) including the possible role of bile acid production, fat digestion, vitamin K content of feces, and vitamin K content of human and cow's milk.

Idiopathic hypoprothrombinemia refractory to vitamin K has been described by Plum (1943a) and by Murphy and Clark (1944). Thorough investigations on the technique for prothrombin determination were made by Plum and Larsen (1942).

Statistics on the value of vitamin K treatment of mothers prior to delivery on the incidence of bleeding and the death rate among the newborn have been published by Beck *et al.* (1941), by Hellman and Shettles (1942), and by Parks and Sweet (1942). Statistics on the treatment of the infant were published by J. Lehmann (1944) and others.

Further investigation on the successful use of vitamin K in gastrointestinal diseases of children has been published by Plum (1943b). Bercovitz and Paye (1944) reinvestigated the occurrence of vitamin K deficiency in case of ulcerative colitis (Clark *et al.*, 1939). They confirmed that vitamin K deficiency may be found in cases of this disease.

The assertion that vitamin K deficiency occurs in patients with pulmonary tuberculosis (Levy, 1942; Sheely, 1941) was reinvestigated by Plum and Poulsen (1942) but was not confirmed, a fact which is in accordance with preliminary investigations by Gyntelberg and Dam (1941).

Marie Stoppelman (1942) reported that vitamin K increases the agglutinating power of serum and argued that blood transfusions should not be given until 48 hours after administration of vitamin K. Subsequent investigators failed to confirm this claim (Gammelgaard *et al.*, 1943; Tronsberg, 1944).

Kinsey (1944) recommends the parenteral ingestion of menadione by blood donors, up to 18 mg. 24 hours before, or 20–30 mg. 48 hours before donation to patients with intrahepatic jaundice although it is stated that the prothrombin content in the blood of the donors remained essentially the same before and after they received vitamin K.

2. *Hypoprothrombinemia of Pancreatic Origin*

The influence of the pancreas on prothrombin was first studied by Sproul and Sanders (1941) in experiments with cats. The pancreatic duct was cut or pancreatectomy was performed. Hypoprothrombinemia developed in the course of some time. Begtrup and Tage-Hansen (1947) studied plasma prothrombin in dogs before and after pancreatectomy. They found that this form of hypoprothrombinemia was refractory to vitamin K therapy, that it developed about 1 week after the operation was performed and disappeared spontaneously about 3 weeks after the operation. Begtrup (1947, 1948) found a case of pancreatic disease associated with hypoprothrombinemia which could not be alleviated by vitamin K therapy. The patient suffered from a pancreatic tumor, was not jaundiced, and did not show signs of liver damage in the usual tests. The prothrombin was about 40% of the normal value.

3. Diagnostic Application

Hypoprothrombinemia may occur in patients with jaundice due to obstruction as well as in those with jaundice due to hepatitis. In obstructive jaundice the prothrombin will rise to normal values after ingestion of vitamin K in suitable form and quantity whereas in hepatitis the response is only slight. Lord and deWitt Andrus (1941) based a test for liver function on this fact and used it for discrimination between the two types of jaundice. Many other investigators have studied the value of this diagnostic procedure (Koller, 1941; Kark and Souter, 1941; Begtrup and From Hansen, 1942; From Hansen and Begtrup, 1943; Stein, 1944). Kark and Souter presented a detailed discussion of the possible variations of the response in the test and their diagnostic significance. Begtrup and From Hansen stressed the fact that it is unnecessary to give, orally or parenterally, more than 1 mg. of vitamin K preparation such as menadione bisulfite or compounds of similar activity. If the increase in prothrombin 24 hours after the ingestion is 20% of the initial value or less, it is safe to conclude that damage of liver parenchyma is present. Uncomplicated cases of obstruction will give a greater response (usually $> 50\%$) in the same length of time.

4. Suggested Therapeutic Applications in Cases without Generally Recognized Hypoprothrombinemia

The most extensive use of vitamin K therapy has been proposed by A. Palladin (1945). He recommends the use of menadione (10–15 mg. in 0.1% alcoholic solution diluted with an equal amount of water before use) in cases of hemorrhages which are not (or not unanimously) considered due to low prothrombin, such as hemorrhages from granulations, pulmonary hemorrhages from penetrating wounds of the thorax, or from pulmonary tuberculosis; prophylactically against bleeding in connection with tonsillectomy; sequestrectomy in osteomyelitis of maxilla following gunshot wounds; further against bleeding from fresh open wounds, bed sores, frostbites, burns, ulcerous stomatitis. According to Palladin such treatment accelerated healing, shortened convalescence and enabled soldiers to return to the front more speedily; it also influenced capillary hemorrhages present in scurvy. Palladin considers it dangerous to limit the use of vitamin K to cases with low prothrombin and suggests that vitamin K counteracts bleeding in other ways than by raising prothrombin, possibly by raising fibrinogen (cf. Field and Dam, 1946). The reviewer has not been able to find any convincing proof for these sweeping statements and they are not generally accepted as yet. Goldberg and

Milgram (1945) argue the use of vitamin K in cases of hemorrhages after extraction.

Kallós (1946) reports increased prothrombin time in patients with chronic urticaria and angioneurotic edema in whom allergic etiology could not be demonstrated, and mentions that vitamin K therapy seems to have a beneficial effect. These statements still await further investigation.

Vitamin K therapy in menorrhagia has been discussed by Gubner (1944), its use in the treatment of chilblains by Wheatley (1947).

The possible use of the antibiotic compound 2-methyl-4-amino-1-naphthol hydrochloride in clinical treatment of dermato-mycoses has been suggested (Pratt *et al.*, 1948).

A postulated relation of vitamin K and vitamin K like substances to *dental* problems has been extensively discussed in the literature. Although the reviewer is of the opinion that the results extend beyond the field of vitamin research, it might be of interest to mention some of the contributions which have appeared.

In 1942 Fosdick *et al.* (1942) reported that 1 mg. of menadione in 100 cc. of a saliva-glucose mixture prevented acid formation in a 4-hour incubation period, whereas the same mixture in absence of vitamin K produced up to 2 milliequivalents of acid under the same conditions. In clinical observations the authors found that menadione, in the same concentrations as in the *in vitro* experiments, also inhibited acid formation. The authors suggested that such an inhibitory action of vitamin K was not due to any antiseptic property, and proposed the use of vitamin K in the prevention of dental caries. Armstrong and Knutson (1943) and Armstrong *et al.* (1943), comparing the inhibitory action of 13 quinones, with high, low, and no vitamin K activity, in conditions similar to those in Fosdick's experiments, demonstrated that the inhibitory effect of the various quinones on acid production was due to the bacteriostatic and bactericidal action of the quinone structure, independent of any vitamin K activity. Further, they showed that several of the quinones effective in the prevention of acid formation in incubated saliva-glucose mixtures also inhibited the growth of certain cocci.

Fancher *et al.* (1944) tested the effect of certain substances, including most of the known vitamins, on the rate of acid formation from fermentable sugars in saliva. Only cholesterol, "Cerophyl," and thiamine stimulated acid formation. Both menadione and natural vitamin K_1 inhibited acid formation. There was no indication that this action was related to the vitamin K activity of the compounds. Calandra *et al.* (1944) studied the action of certain derivatives of the quinones on the rate of acid formation in saliva, and all those tested inhibited acid forma-

tion in varying degrees. Menadione proved to have an inhibitory power equal to any of the other quinones tested, and superior to most of them. The mechanism whereby the quinones inhibit acid production and act as antibiotic agents (Geiger, 1946; Hoffman-Ostenhof, 1947) is very complex.

Granados and Dam (1945) studied the influence of vitamin K on the rat incisor, using basal diets containing 1% sulfadiazine for preventing vitamin K production by the intestinal flora. No gross changes in the incisors could be attributed to vitamin K deficiency. A striking growth decrease and a marked decline of eruption and attrition rates were produced by the sulfadiazine content of the rations.

Hodge (1944) and Hatton *et al.* (1945) reported that in albino rats menadione did not decrease the number of carious lesions. In humans, Burrill *et al.* (1945) studied the effect of menadione incorporated into chewing gum, on the occurrence of new carious lesions, and the progress of old ones. In the subjects who chewed the vitamin K containing gum there was a reduction of 60 to 90% in the occurrence of new lesions, but no apparent inhibition in the progress of caries already present. However, the Professional Service Schools (1946) found no differences in the number of new carious lesions exhibited by subjects who had chewed menadione-containing gum as compared with the controls that chewed menadione-free gum.

Thus, though certain quinones, regardless of their vitamin K activity, inhibit the acid formation by, and growth of, certain cocci, menadione does not decrease the incidence of caries in the albino rat, and in humans the results are contradictory. The basis for these contradictions could, at least partially, lie on a misconception of the caries process itself. The optimistic concept on the beneficial role of vitamin K, through its acid-inhibiting power, in dental caries, has been based, so far, on the assumption that the initial lesion is fundamentally a process due to acid action (Fosdick, 1943). The experimental and clinical studies carried out up to the present, do not support this view. The work of certain investigators, reviewed recently by Pincus (1944), Nuckolls and Frisbie (1946), Leicester (1946), and Gottlieb (1947) on the importance of the organic component of the enamel in the initiation of caries, deserves consideration. Any further study of the problem in animal experiments should be carried out with animals that are more suitable for the dietary development of dental caries than the albino rat.

VIII. Summary

The review deals mainly with developments since 1941.

Some new vitamin K compounds have been reported.

A series of color reactions more or less specific for vitamin K active

substances have been proposed but here has been little progress towards making these reactions applicable to biological material.

An elaborate theory for the participation of vitamin K in prothrombin formation has been set forth without convincing experimental proof. The relation of vitamin K to the more advanced concept of blood coagulation has been discussed by some authors.

It has been suggested that vitamin K might act on other biological processes than prothrombin formation. Some of the more promising of the studies within this field seem to be those dealing with the influence of vitamin K-like substances on metabolic processes in tissue or individual cells.

The formation of vitamin K by bacteria has received renewed attention through the demonstration of the blockage of this process by dihydroxystearic acid and, possibly, by massive doses of vitamin A.

The development of hypoprothrombinemia by other means than deprivation of vitamin K as well as the influence of vitamin K and its derivatives on such hypoprothrombinemias has been studied by many authors. Dicumarol and isosteres of vitamin K with antagonistic action have been dealt with extensively. According to several authors the mode of action of dicumarol does not seem to be a simple "replacement antagonism."

The well established therapeutic application of vitamin K in cases with generally recognized hypoprothrombinemia has been studied in detail by many investigators. The same applies to the diagnostic application in liver function tests. In addition thereto a considerable number of medical applications have been suggested in cases without hypoprothrombinemia. The reviewer emphasizes the lack of sufficient evidence for the relevance of vitamin K therapy in many of these cases.

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Nutritional Requirements of the Cotton Rat and Hamster*

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CONTENTS

I. Introduction

In the course of the development of the science of nutrition, it became readily apparent that the dietary requirements of animals varied for different species. The use of a number of different species therefore, greatly facilitated the discovering of nutritional factors, particularly the B vitamins, some of which are needed as dietary factors by one species but not by others. These studies were confined largely to the mouse, rat, chick, dog, monkey, and guinea pig. In recent years considerable interest has developed in studying the nutritional requirements of two other mammalian species, the cotton rat and the hamster. These animals have been widely used in virus studies and more recently in studies on the influence of diet on the incidence and extent of dental caries. Undoubtedly, the importance of these observations has prompted interest in studies on nutritional requirements.

* A portion of this material was presented at the Symposium on "Vitamin Requirements of Laboratory Animals," Division of Agricultural and Food Chemistry of the American Chemical Society, September, 1946.

II. Nutritional Requirements of the Cotton Rat

1. General Considerations

The cotton rat is more excitable than the white rat and certain precautions are necessary in the care of these animals, particularly in the care and management of the stock colony. The breeding females are usually housed in individual cages and the young are disturbed as little as possible during the first few days of life. The gestation period is about 28 days and the litter size varies from 3 to 11. Further information on typical equipment and management procedures has been reported by Meyer and Marsh (1943), Meyer (1942), McIntire, *et al.* (1944) and Meyer and Meyer (1944).

Several studies have been made demonstrating the susceptibility of the cotton rat (*Sigmodon hispidus hispidus* and *Sigmodon hispidus littoralis*) to St. Louis encephalitis, Lansing strain of poliomyelitis, and to diphtheria toxin (Armstrong, 1939; 1940; Jungeblut, 1940). Other studies in which the levels of certain vitamins were varied (Weaver 1945, 1946) failed to reveal any relationship in the level of vitamins fed and the susceptibility to poliomyelitis virus.

Following the demonstrations of the importance of the cotton rat in virus studies, investigations were initiated to determine its dietary requirements, particularly of the B vitamins. Most of the work has been done with *Sigmodon hispidus hispidus*, consequently subsequent work to be reviewed refers to this species unless otherwise indicated. In the earlier work (McIntire *et al.*, 1944) a purified ration was devised for studies on the B vitamin requirements. In subsequent studies (Schweigert, *et al*, 1945; Schweigert, 1947) the composition of the purified ration has been modified somewhat, specifically, the casein level has been increased from 18 to 24% and biotin and pteroylglutamic acid, which have recently become available, have been added. The composition of this purified ration is shown in Table I. For growth studies, the young were weaned at 15–20 days of age at an initial weight of 15–25 g.

Animals grow well when fed the purified diet indicated; however, the rate of growth is increased when liver concentrates are included in the ration (McIntire *et al.*, 1944; Schweigert *et al.*, 1945). Since these animals have not been inbred extensively, particular attention must be given to equal distribution of the young from each litter among the different dietary treatments in each experimental series. The growth rates of the males are more rapid than the females and should be taken into account in comparing the results obtained, particularly if equal numbers of females and males are not included in each group (Schweigert *et al.*, 1945; Anderson *et al.*, 1948).

TABLE I

Composition of Purified Ration Suitable for Growth Studies with the Cotton Rat

Basal components	Per cent	Vitamins	Mg./100 g.
Purified casein	24	Thiamine hydrochloride	0.25
Sucrose	67	Riboflavin	0.30
Salt mixture[1]	4	Pyridoxine hydrochloride	0.25
Corn oil (Mazola)	4.7	Ca panthothenate	2.0
Fortified cod liver oil[2]	0.3	Niacin	2.5
		Choline chloride	100.
		Pteroylglutamic acid	0.2
		Biotin	0.01
		p-Aminobenzoic acid	30.
		Inositol	100.

[1] Hegsted *et al.* (1941).
[2] Nopco XX (3000 I.U. of Vitamin A and 400 I.U. of Vitamin D/g.).

2. Vitamin Requirements

The qualitative requirements for thiamine, riboflavin, pyridoxine (vitamin B_6), Ca pantothenate, niacin, biotin, choline, inositol, *p*-aminobenzoic acid, and pteroylglutamic acid (folic acid) have been determined, as well as information on the quantitative requirements for thiamine, riboflavin, pyridoxine and Ca pantothenate (McIntire *et al.*, 1944; Schweigert, 1947). A summary of these results and for the white rat is shown in Table II. The symptoms of the deficiency for thiamine, riboflavin, pantothenic acid and vitamin B_6 were similar to those observed for the white rat. It is also of interest that the amounts of these vitamins required for optimum growth of the cotton rat are similar to those required by the white rat. It should be pointed out that these requirements are tabulated for studies conducted with diets of similar composition, in that variants in the diet are known to influence the dietary requirements. The results presented here, however, are useful for comparative purposes.

It will be noted that the qualitative requirements are similar, with the exception of inositol. Also, the response noted when comparable diets are used is greater with the addition of niacin or liver concentrates for the cotton rat than for the white rat. The dietary dispensability indicated for several of the vitamins is based on observations that the addition of the factor to the diet does not increase the rate of growth.

In Table III detailed results obtained with inositol, choline, and liver extracts are presented. It will be noted that the omission of inositol resulted in a slow rate of growth, while the omission of choline resulted in a less marked retardation in growth as compared to the

TABLE II

B Vitamin Requirements for the Cotton Rat and White Rat for Growth
(Mg./100 g. ration)

Vitamin	Quantitative	
	Cotton rat	White rat
Thiamine	.15	.08–.15
Riboflavin	.08 > < .30	.10–.15
Pyridoxine	.10	.08–.10
Ca pantothenate	.80	.80
	Qualitative	
Niacin[1]	±	±
Biotin	–	–
Pteroylglutamic acid	–	–
Inositol	+	–
Choline	+	+
p-Aminobenzoic acid	–	–
Unknown factors	+	+

[1] The response to niacin supplementation is dependent on the tryptophan content of the diet (see text).

TABLE III

Effect of Supplements of Inositol, Choline, and Liver Extract on the Growth of Cotton Rats
(Six weeks growth period used)

Dietary regimen[1]	No. of animals	Grams gain per week
Inositol basal	14	2.9
Basal + 0.1% inositol	6	5.9
Basal + 0.1% inositol + .03% *p*-aminobenzoic acid	34	5.6
Choline basal	14[2]	6.7
Basal + 0.1% choline	12	8.0
Purified diet	32	6.9
Purified diet + 4% liver extract[3]	31	9.0
Dog food stock ration	13	9.0

[1] The diets described as basal + 0.1% inositol, basal + 0.1% choline, and purified diet contain a complete vitamin supplement and are identical or similar to the diet described in Table I.

[2] Five of the animals died prior to 4 weeks. The results for choline were obtained at the end of 4 weeks on experiment.

[3] A water soluble concentrate prepared from dried, defatted liver; Wilson Laboratories, Chicago, Illinois.

respective control groups. In later work, less striking differences were obtained in the rate of growth when inositol was omitted. Nevertheless, it does indicate that the requirement for inositol is different than for the white rat.

Recent studies with several species of animals, including the cotton rat, have shown that tryptophan can be converted to niacin. In brief, if sufficient tryptophan is provided either as protein or as the free amino acid, the apparent dietary requirements for niacin can not be demonstrated and an increase in the excretion of niacin derivatives occurs (Schweigert *et al.*, 1947; Schweigert and Pearson, 1948). A summary of the influence of the level of casein on the response to niacin supplementation is given in Table IV. It will be noted that when less than 24% casein is included in the diet and when a protein deficient in tryptophan (gelatin) is fed, that an increased growth performance and survival results when niacin is included in the diet. It is concluded, therefore, that the cotton rat, like the white rat, can utilize tryptophan to form niacin provided that sufficient tryptophan is present in the diet over that needed for protein synthesis.

TABLE IV

Effect of Level of Casein (Tryptophan) on the Dietary Requirement of the Cotton Rat for Niacin

Dietary regimen[1]	Growth rate (g./week)		Mortality of unsupplemented group
	Without niacin	With niacin[2]	
12% Casein	1.7	5.6	5 out of 10 dead at 4 weeks
18% Casein	5.7	7.1	4 out of 16 dead at 6 weeks
24% Casein	8.7	8.0	None out of 7 dead at 6 weeks
12% Casein and 12% gelatin	3.0	5.6	2 out of 6 dead at 4 weeks

[1] These diets are modifications of the diet described in Table I with the casein content varied at the expense of sucrose and niacin either omitted or added at the level indicated.

[2] 1.0 mg. niacin added per 100 g. of ration.

In earlier work (McIntire *et al.*, 1944) 18% casein was used and, in accord with the work summarized here, a growth response was observed with niacin supplementation. It is apparent from subsequent work, however, that although the rate of growth of the white rat is increased only slightly when an 18% casein diet is fed, that the requirements for niacin for the cotton rat and white rat are qualitatively similar.

From the evidence presented in Table III, and from other data

(McIntire *et al.*, 1944; Schweigert *et al.*, 1945) it is clear that a marked growth response is observed when the purified diets are supplemented with liver extract, a good source of unknown factors. The magnitude of the response noted with the addition of liver extract is much greater than is obtained with the white rat. Although some preliminary studies have been done to fractionate the liver extract (Schweigert *et al.*, 1945) progress has been very slow due to the heterogeneity of the stock animals and the limited number of animals available.

The cotton rat does not require vitamin C for growth (Clark and Jungeblut, 1940) but does develop diarrhea and hemorrhages of the gingivae when 10% of glucoascorbic acid, an antimetabolite of ascorbic acid, is included in the diet (Woolley and Krampitz, 1943). These effects were noted in 5 days; however, no effects were noted when 2% glucoascorbic acid was fed for a period of 10 days. Information is not available on the requirements of the cotton rat for minerals or for the fat-soluble vitamins.

3. Miscellaneous Growth Studies

A high incidence and extent of carious lesions occur in the molars of cotton rats fed diets similar to the purified diet listed in Table I (Shaw *et al.*, 1944a and b; Schweigert *et al.*, 1945; Schweigert, Shaw *et al.*, 1946; Schweigert, Potts *et al.*, 1946; Anderson *et al.*, 1947, 1948). While few or no carious lesions develop in the white rat when such diets are fed, the cotton rat will develop from 20 to 40 cavities after 14 weeks on experiment. It is of particular interest that these diets permit an excellent rate of growth. When fermentable carbohydrates are fed the caries incidence is high; the incidence can be reduced by feeding starch or dextrin as the carbohydrate, by feeding a high-fat diet, milk as the sole diet, and other changes in the dietary treatment. These observations offer an excellent opportunity to study experimentally dietary factors that influence tooth decay.

It is of interest that the growth rate and survival is greatly reduced when starch or finely ground dextrin is fed as the carbohydrate. The animals scatter the food and fail to thrive. These results are in contrast to observations made with the white rat.

A high incidence of trichobezoar has been observed when purified diets were fed to cotton rats (Howell *et al.*, 1948) which could be prevented by including 10% cellulose in the diet.

4. Reproduction and Lactation

A few studies have been made on the influence of diet on reproduction and lactation performance (Schweigert, 1947, 1948). Since these

animals are very excitable and the mortality is high when they are mated as adults, the difficulties inherent in these studies are much greater than with the white rat. Some improvement can be obtained by placing the male and one or two females in a cage at weaning age. This technique, therefore, involves the influence of the diet on the performance of the male as well as the female, since both receive the same diet from an early age. Quite frequently pairs fail to produce litters even when fed stock rations, while others will raise excellent litters at regular monthly intervals. The reasons for this variation cannot be readily explained.

Some results obtained in preliminary studies are summarized in Table V. The stock pairs are disturbed as little as possible and one cannot readily obtain complete records on the performance. Nevertheless, the results indicate that fewer pairs produce young when purified rations are fed and, for the 8 months' period summarized, fewer pairs

TABLE V

Effect of Diet Composition on the Reproduction of the Cotton Rat

Dietary regimen	No. pairs producing young	No. litters produced (total)	No. young per litter (average)
Stock ration[1]	3 out of 4	21	4.5
Purified rations[2]	4 out of 16	5	2.0
Purified rations + 4% liver extract	5 out of 7	16	2.6

[1] Rockland rat pellets, complete.

[2] A modification of the purified ration indicated in Table I. The casein level was increased to 30% at the expense of the sucrose, and the amount of vitamin A and D concentrate and the B vitamins was doubled with the exceptions of niacin, choline, inositol, and *p*-aminobenzoic acid.

produce successive litters. An improvement in the performance was obtained when liver extract was added, but the average size of the litters was considerably below that obtained for animals fed stock ration. The weights of the young at weaning were similar for all groups. These results indicate that although reproduction can be obtained with the use of a purified diet, more dietary factors are needed for optimum reproduction and lactation. It is recognized that the purified diets used favor the development of dental caries, and this may have a complicating effect in studies of this type. The animals eat readily, however, and reach a mature weight approximating that obtained when stock ration was fed.

III. Nutritional Requirements of the Hamster

1. General Considerations

The Syrian or golden hamster (*Cricetus auratus*), like the cotton rat, has been an extremely valuable animal for public health work in that

it is susceptible to viruses and tubercle bacilli infections (Lennette, 1941; Broun *et al.*, 1941; Wheeler and Nungester, 1942; and Griffith, 1939). The incidence and extent of tooth decay are much higher in the hamster than in the white rat, but lower than in the cotton rat (Arnold, 1942; Keyes, 1946).

Difficulties in working with these animals are similar to those for the cotton rat. Descriptions of the care and management of colonies have been reported (Laidlaw, 1939; Hamilton and Hogan, 1944). The rate of growth and final weight of the hamster are somewhat less than that of the cotton rat. The gestation period is 16 days and the litter size ranges from 3 to 8. A purified ration has been devised for the hamster which supports growth rates comparable to those obtained when stock rations are fed (Hamilton and Hogan, 1944). The composition of this ration is given in Table VI. The cellular and chemical composition of the blood have been studied (Rose, *et al.*, 1946).

TABLE VI

Composition of Purified Ration Suitable for Growth Studies with the Hamster

Basal components	Per cent	Vitamins	Mg./100 g.
Purified casein	20	Thiamine hydrochloride	0.8
Cerelose[1]	65	Riboflavin	1.6
Lard	7	Pyridoxine hydrochloride	1.2
Cellulose	3	Ca pantothenate	1.0
Salt mixture[2]	4	Niacin	5.0
Vitamin A–D mixture[3]	1	*p*-Aminobenzoic acid	100
		Inositol	250
		Choline chloride	400
		2-Methyl-1, 4-naphthoquinone	3.0
		α-tocopherol	2.5

[1] Glucose monohydrate.
[2] Detailed composition given in a paper by Hamilton and Hogan (1944).
[3] A mixture of oleum percomorphum and lard which supplied 1200 I.U. of vitamin A and 170 I.U. of vitamin D per g.

2. Vitamin Requirements

With the use of basal rations similar to that described in Table VI, Routh and Houchin (1942), Cooperman, *et al.* (1943), and Hamilton and Hogan (1944), have studied the qualitative requirements of the hamster for various vitamins. Their results are summarized in Table VII.

The omission of thiamine, riboflavin, pantothenic acid, or pyridoxine from the diet resulted in the production of deficiency symptoms similar to those observed in the white rat (Routh and Houchin, 1942; Hamilton and Hogan, 1944).

All three groups have studied the requirement for niacin. Routh

and Houchin (1942) reported that a marked weight loss, alopecia, and death occurred when niacin was omitted from the diet and these effects could be eliminated by the administration of 100 γ of niacin/day. The other investigators reported no deleterious effects when this vitamin was omitted. Cooperman *et al.* (1943) and Hamilton and Hogan (1944) used 18 and 20% casein diets, respectively, and from data obtained with other animals, it would appear that these amounts of casein should be approximately enough to eliminate the need for added niacin. Unfortunately,

TABLE VII

Qualitative Vitamin Requirements for the Growth of Hamsters

Vitamin	Qualitative requirement	Remarks on the deficiency and reference
Thiamine	+	Polyneuritis in 12 days[1,2]
Riboflavin	+	Death in 20 days[1,2]
Calcium pantothenate	+	Death in 20 days[1,2]
Pyridoxine (vitamin B_6)	+	Death in 24 days[1,2]
Niacin	±	Slow growth[1]
		No effect[2,3]
Biotin	±	Slow growth[3]
		No effect[2]
p-Aminobenzoic acid	−	No effect[2]
Inositol	−	No effect[2]
Choline	−	No effect[2]
Vitamin K	+	Slow rate of growth, hemorrhage[2]
Vitamin E	+	Collapse and death in 4–18 weeks[2,5]
Vitamin C	−	No effect[3,4]

[1] Routh and Houchin (1942).
[2] Hamilton and Hogan (1944).
[3] Cooperman, *et al.* (1943).
[4] Clausen and Clark (1943).
[5] Houchin (1942a).

the level of casein used by Routh and Houchin was not given.[1] It seems likely that this factor may explain at least in part the discrepancies reported. Cooperman and associates attributed the difference to the fact that Routh and Houchin's work was complicated with a biotin deficiency. Later work by Hamilton and Hogan, however, indicated that neither niacin nor biotin was needed for optimum growth. Likewise, the latter workers observed that choline, inositol, and *p*-aminobenzoic acid were not required. Cooperman *et al.* reported better growth when the latter two substances were fed. As more work is done with the

[1] Dr. Routh (private communication) indicated that 20% of purified casein was used in these studies and that in later experiments a growth response with niacin supplementation was not consistently obtained.

hamster and more information is accumulated on the interrelationships of these factors, explanations for these apparent differences in results may be forthcoming. The role of pteroylglutamic acid (folic acid) is not known since it was not available when these studies were carried out.

The need for vitamins E and K during the growth period has been shown by Hamilton and Hogan (1944). The need for vitamin K by the hamster is of particular interest since the white rat does not require a dietary source due to the fact that sufficient amounts of this factor are synthesized by intestinal microorganisms. A somewhat slower rate of growth and hemorrhage occurred when vitamin K deficient diets were ingested. The animals fed vitamin E deficient diets collapsed and died in 4 to 18 weeks. If the animals were given α-tocopherol shortly after collapse they exhibited a rapid recovery. Similar results with vitamin E deficient diets have been reported by Houchin (1942a). In this and other studies changes in the oxygen consumption, chloride and creatine content of the tissues have been determined (Houchin, 1942b; Houchin and Mattill, 1942a and b).

The hamster, like the cotton rat and white rat, does not require vitamin C (Clausen and Clark, 1943; Cooperman *et al.*, 1943).

3. Reproduction and Lactation

Hamilton and Hogan also studied the requirements of the hamster for reproduction and lactation, and found that niacin, choline, and inositol were helpful although they could not demonstrate a need for these vitamins during the growth period. Further work was indicated to establish the essentiality of biotin or *p*-aminobenzoic acid. Only fair results were obtained when the following B vitamins were included: thiamine, riboflavin, vitamin B_6, niacin, pantothenic acid, choline, biotin, inositol, *p*-aminobenzoic acid. It is, therefore, apparent that the requirements for satisfactory reproduction are not completely known. Some of their results are shown in Table VIII. The results obtained with inositol-deficient diets are particularly striking. It is possible that those obtained on reproduction with the purified diets may be improved somewhat by increasing the level of protein above 20%, or by the inclusion of pteroylglutamic acid. The nature of the factors involved, as in the case of the cotton rat, are not known.

IV. General Comments

It is of particular interest to accumulate knowledge on the nutritive requirements of the cotton rat and hamster, since these animals are considered to be less susceptible to infection than the guinea pig, but much more susceptible than the laboratory white rat. The possible

TABLE VIII

Effect of the Composition of the Diet on the Reproduction and Lactation Performances of the Hamster

Description of diet[1]	No. of litters	No. of young per litter (average)	No. of litters weaned	Comments
Stock	38	5.3	31	
Purified	5	5.4	5	Failed to produce successive litters, weaning weights equivalent to group fed stock ration
Biotin deficient	19	3.7	17	Somewhat lower weaning weights than for the group receiving biotin
Choline deficient	12	4.0	4	Low weaning weights
Niacin deficient	7	2.8	4	Low weaning weights
Inositol deficient	25	...	1	Several females died during parturition, many young born dead or failed to survive the 1st week of life

[1] The purified diet is the same as that described in Table VI and the vitamin deficient diets were prepared by omitting the appropriate vitamin from this diet.

relationship between their comparative nutritional requirements and their susceptibility to viruses and dental caries is a matter of speculation. With the information on the good growth performances obtained with the purified rations listed in Tables I and VI and partial success with reproduction using similar rations, studies on virus susceptibility, etc., can be made with the use of diets with defined composition. Much more work is needed, however, in expanding the knowledge now available on the requirements of the cotton rat and hamster not only for growth, but for reproduction and lactation as well.

V. Summary

Satisfactory growth of the cotton rat and hamster has been obtained with the use of purified diets.

The quantitative requirements of the cotton rat and hamster for thiamine, riboflavin, pyridoxine (vitamin B_6), and pantothenic acid are similar to those of the white rat. Vitamin C is not required by either species.

The cotton rat grows at a more rapid rate when inositol is included in the diet. The response to niacin supplementation is influenced by

the protein (tryptophan) intake. Biotin, pteroylglutamic acid, and *p*-aminobenzoic acid are not needed in the diets for the normal growth of the cotton rat. Additional factors needed for growth and reproduction are present in liver extract.

For the hamster, the evidence is not conclusive on the essential nature of dietary sources of niacin, choline, biotin, inositol, and *p*-aminobenzoic acid for growth. Of particular importance is the demonstration that the hamster requires a dietary source of vitamin K.

Preliminary studies on the reproduction and lactation performances of the cotton rat and hamster when fed various purified diets have been presented.

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Vitamins as Pharmacologic Agents

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CONTENTS

I. Introduction

"Experimental pharmacology, in its broadest meaning, deals with the response of living organisms to chemical agents; or expressed in different words, examines the behavior of the organism in a chemically altered environment." This definition by Hans Horst Meyer, one of the founders of modern experimental pharmacology, clearly indicates the difficulties facing the reviewers of a topic as broad as "Vitamins as Pharmacologic Agents." Their task is complicated by the fact that the scientific literature provides relatively little assistance in the form of previous articles along similar lines. Indeed, while the fields of vitamin chemistry, biochemistry, physiology, pathology and, of course, therapy have been covered ably and thoroughly, it seems that the only earlier review of this topic was that presented by one of us (Molitor) in 1942 at a

symposium of the American Pharmacological Society. This is unfortunate because the selection of individual papers for inclusion in such a review is by necessity arbitrary and depends upon the reviewer's personal interpretation of the term "pharmacology."

Obviously, it is impossible to include more than a small fraction of the many hundreds of papers dealing with one of the many aspects of the topic; and thus the imposition of arbitrary limitations becomes unavoidable.

If we adopt Hans Horst Meyer's definition of pharmacology, this treatise would describe the pharmacologic effects of vitamins in healthy as well as in diseased states. However, since the therapeutic use of vitamins in the correction of a specific deficiency has been repeatedly reviewed, we feel justified in limiting the discussion to the effect of vitamins on normal animals and in diseases other than avitaminoses. In such cases the doses required are generally far in excess of those therapeutically effective in specific vitamin deficiencies. Such effects are, for example, the curare-like paralysis in frogs (Demole, 1938a); or the excessive salivation, nausea, vomiting, and collapse produced in dogs by intravenous injection of 50 mg./kg. of thiamine (Molitor, 1942).

Another nonspecific effect of a vitamin is the vasodilator action of niacin (Sebrell and Butler, 1938; Loman *et al.*, 1941; Positano and Ruggieri, 1942). However, one would not be justified to regard this as a pharmacologic property of niacin, *the vitamin*, since this effect is not produced by niacinamide (Field and Robinson, 1940) which otherwise fully shares the specific vitamin effects of niacin. Similarly, 2-methyl-1,4-naphthoquinone may produce severe skin irritation in sensitive individuals (Page and Bercovitz, 1942). Nevertheless this property is common to many compounds to which this quinone is not related structurally.

Other purely toxic effects of vitamins are those of pyridoxine, which in excessive doses produces clonic-tonic convulsions (Unna, 1940); or of riboflavin, which upon repeated intravenous injection of large doses is precipitated in the glomeruli, thus mechanically interfering with urinary excretion (Unna and Greslin, 1942). None of these "toxic" effects, however, are of even remote practical significance since the doses necessary to produce them are far beyond those therapeutically employed.

Any vitamin may be expected to restore to normal, functional changes which result from the deficiency of that vitamin. It is not easy, however, to determine whether therapeutic results obtained by administration of a vitamin in diseases not following the pattern of a typical deficiency are ascribable to properties other than those necessary for the function as a vitamin.

For example, the therapeutic effects of thiamine in cardiac failure of advanced beriberi (Weiss and Wilkins, 1937) or of niacinamide in psychosis of pellagra (Frostig and Spies, 1940) are recognized as the correction of typical vitamin deficiency states. We are not, however, in a position to state whether the therapeutic activity of pyridoxine in radiation sickness is a specific *vitamin* effect (Maxfield *et al.*, 1943), since it is unknown whether intense radiation produces a state of acute pyridoxine deficiency. The effect of large doses of vitamin D in rheumatic arthritis (Wyatt *et al.*, 1936; Vrtiak and Lang, 1936) or of ascorbic acid in asthma (Hagiesco *et al.*, 1938; Diehl, 1937) and hay fever (Holmes and Alexander, 1942) may serve as similar examples, provided that the alleged value of vitamin therapy in these conditions withstands critical examination.

As mentioned before, the amount of a vitamin necessary to produce "non-specific" therapeutic effects, is usually much larger than that needed to correct a deficiency, although it is still well below the toxic range. In turn, the doses needed to cure a fully developed vitamin deficiency state greatly exceed those sufficient to maintain an organism in optimum nutritional condition.

The great difference in effective doses in normal and in pathologic conditions need not be surprising, since it is well known that the optimal condition for demonstration of a drug effect is a state of stress and unbalance. Antipyretics, cardiacs, antispasmodics, analgesics, etc. may serve as examples for this.

Certain effects of vitamins cannot be observed by simple inspection or application of the commonly used pharmacologic technics. Thus, Porter *et al.* (1947) have shown that in rats the only change produced by the administration of desoxypyridoxine (a vitamin analogue of pyridoxine) is an increase in the urinary excretion of xanthurenic acid. While a metabolic effect such as this cannot be termed pharmacologic, it might well indirectly alter the conditions required for the action of other drugs. Seeler (1945) observed that administration of pyridoxine to malaria infected birds abolished the curative effect of quinine or atabrine, although in the dose used pyridoxine failed to produce visible effects when injected in either normal or infected birds. Similarly, *p*-aminobenzoic acid inhibits in normal animals the effect of sulfa drugs; and vitamin K prevents the appearance of the hemorrhages following administration of sulfaquinoxaline (Seeler *et al.*, 1944a) or dicumarol (Davidson and MacDonald, 1943; Lucia and Aggeler, 1944).

On the basis of the foregoing considerations, we feel inclined to include in this review vitamin actions which are pharmacodynamic in character, regardless of whether they occur in normal or deficient animals; we shall omit, however, a discussion of those pharmacologic effects of

vitamins which constitute a restoration to normal of a typical vitamin deficiency.

The comparatively minute dose of a vitamin needed to cure a specific deficiency, contrasted to the much larger dose necessary to produce an effect in the normal animal or in one affected with a disease not caused by a vitamin deficiency, often permits a diagnosis on the principle "post hoc ergo propter hoc"; indeed, several pathologic conditions of hitherto unclear etiology have been elucidated by their dramatic response to small doses of vitamins. We have in mind particularly the neurologic and psychiatric disorders associated with deficiencies of the B complex, for example the Wernicke-encephalopathy (paralysis of the eye muscles, balance disturbance, and drowsiness, usually terminating in fatal coma) and the Korsakoff psychosis (delirium, confabulation, loss of memory without loss of consciousness). However, while many cases of this psychiatric disorder, as well as of delirium tremens, promptly respond to relatively large (10–50 mg.) doses of intravenously administered thiamine (Bowman *et al.*, 1939; Roch and Sciclounoff, 1938; Caldwell and Hardwick, 1944), there is considerable doubt whether a Korsakoff psychosis etiologically unrelated to thiamine deficiency (such as caused by head trauma or subarachnoid hemorrhage) is influenced by thiamine medication. Indeed, in such cases vitamin administration can serve diagnostic purposes.

An even more striking illustration of the diagnostic value of vitamin administration is the response to B complex vitamins in cases of so-called "subclinical" deficiency diseases. Mental symptoms such as irritability, inability to concentrate, depression, phobias, and anxieties, have been noted as some of the earliest signs of experimentally induced B complex deficiency (Jolliffe *et al.*, 1939; Williams *et al.*, 1940; Sebrell, 1943); prompt relief by vitamin administration may establish a diagnosis which otherwise would be difficult to make.

Another diagnostic use of a vitamin is that of vitamin K in cases of hepatopathy. Since the liver is the site of prothrombin formation Lord and Andrus (1941) have postulated that failure of vitamin K to correct hypoprothrombinemia in jaundiced patients is strong evidence for liver injury; and Turner *et al.* (1944) have successfully used the response to vitamin K as a prognostic criterion in patients suffering from acute hepatitis; and Unger and Shapiro (1948) have used a standard vitamin K tolerance test in estimating the prothrombin response to parenteral administration of large test doses of vitamin K. This test was found to be a sensitive indicator of hepatic disease.

The high specificity of vitamins can be demonstrated in experimentally produced pathologic conditions which closely resemble those

observed in vitamin deficiencies, but are unrelated to them etiologically. Molitor and Sampson (1937) placed a group of rats on a thiamine deficient diet, while pair-fed controls received a complete ration. In both groups, frequent records were taken of body weight, heart rate and sensitivity to the convulsant drug thujone. As the animals lost weight, both groups became more sensitive to the convulsant agent, but only the thiamine deficient group showed the typical bradycardia, which responded within less than 1 hour to an intravenous injection of thiamine.

Failure to respond to vitamin therapy does not justify the conclusion that the pathologic condition is not due to a vitamin deficiency. Factors such as interference with absorption, antagonistic effect of specific vitamin inhibitors, changed physiological conditions (lactation, pregnancy, strenuous work) or an unbalance in the composition of the diet may greatly affect the vitamin requirements. Thus, the average therapeutic dose of niacin or niacinamide in moderately severe cases of pellagra is 25 to 50 mg. by mouth three times daily; fully developed cases of pellagra psychosis, however, require such heroic doses as 100 mg. of niacin intravenously in hourly intervals, up to 1 gram daily (Jolliffe *et al.*, 1940). Similarly the average daily dose of thiamine in uncomplicated cases of thiamine deficiency is 5 to 10 mg. orally, but severe cases of beriberi heart-failure, may require the intravenous injection of 25 to 130 mg. daily (Weiss and Wilkins, 1937). One reason for administering to critically ill patients vitamins by the parenteral route and in doses far above those required for normal maintenance is the impaired absorption often found in deficiency diseases. Another is the belief that very large doses of a drug may exert a "mass effect" capable of turning the tide of a progressing pathologic condition when smaller doses would fail (Williams and Spies, 1938). Since the toxicity of vitamins as medicinal agents is extremely low, no objection would seem to exist against the use of unusually large doses, as long as the administration takes place under medical supervision.

One of the principal obstacles in establishing the cause-and-effect relationship in vitamin therapy is the difficulty of an early diagnosis and quantitative evaluation of vitamin deficiencies. Much progress has been made in the last few years, particularly since the introduction of sensitive and easily performed microdeterminations of the vitamin concentration in the blood, urine, and body tissues (Lowry and Bessey, 1944; Lowry *et al.*, 1945; Lowry and Bessey, 1946). Such methods, however, are not yet available for some of the most interesting vitamins, notably, pyridoxine. Furthermore, it must be borne in mind that the vitamin concentration in the blood is not necessarily an indicator for the state of vitamin saturation of the body as a whole, since the concentration in the

tissues may vastly differ from that in the circulating blood (Youmans *et al.*, 1941). Indeed, even the tissue concentration may vary with the function of the tissue. For example, thiamine is firmly retained in heart and brain at a time when it already has reached dangerously low levels in less vital organs (Ferrebee *et al.*, 1942).

A more indirect method of appraising the adequacy of the vitamin supply is to follow the urinary excretion of a vitamin after administration of a test dose (Abbasy *et al.*, 1935; Mason and Williams, 1942). The theory underlying this approach is that in an organism saturated with vitamin, most of the test dose is likely to be excreted, whereas a depleted organism will retain and store it. It is not possible, however, to estimate solely on this basis the requirements for a vitamin since the differences between the amounts administered and excreted vary with the size of the test dose. Thus, of 100 γ of pantothenic acid given to a rat, 7 γ were excreted (Silber and Unna, 1942); of 250 γ, only 67; and of 3000 γ, only 600. On the basis of these findings the pantothenic acid requirement might have been estimated as 93, 183 and 2400 γ, respectively, while on the basis of growth experiments it actually is about 100 γ. A similar observation has been made in children receiving varying doses of thiamine (Knott, 1936). Increasingly high retentions were found with higher levels: the optimum utilization being at about 100 γ/kg./day.

A still less satisfactory way to estimate the degree of vitamin saturation of the body is the performance of balance experiments and the determination of the amount of vitamins excreted in the feces. It is well known that many vitamins are synthesized by the intestinal bacteria and that this process is influenced not only by the composition of diet, but also by the action of drugs. A diet rich in fat appears to favor the biosynthesis of thiamine (Whipple and Church, 1935) while chemotherapeutic agents such as the sulfonamides and certain antibiotics interfere with biosynthesis (Black *et al.*, 1941; Emerson and Smith, 1945). It must be kept in mind, however, that the vitamins excreted in the feces would not necessarily be available to the organism. For this reason it would seem somewhat risky to judge the state of the body's vitamin stores solely from the outcome of urinary and fecal balance experiments.

As with many other drugs, the mode, rate and frequency of administration are of considerable influence on the pharmacologic and toxicologic effects of vitamins. Adverse reactions are least likely with peroral administration, and most likely with intravenous. This is due partly to the sudden, high drug concentration obtained by the latter mode of administration, partly to the possibility of side-reactions of an allergic (anaphylactic) nature. Thus, the only serious accidents reported with thiamine therapy occurred when it was injected parenterally (Mills,

1941); the signs and symptoms reported were those of an allergic reaction rather than of a specific pharmacodynamic drug effect. In animals, however, it has not been possible to produce such a sensitization. Dogs and guinea pigs injected daily for 10 consecutive days with 50 mg./kg. of thiamine (a dose sufficient to produce severe toxic reactions) failed to show signs of anaphylactic shock, when the animals were challenged after a treatment-free interval of from 10–60 days (Molitor and Seeler, unpublished).

It cannot be emphasized too strongly that experiments of the acute type, viz. single administration of very large doses, have but little value for the appraisal of the toxic properties of a vitamin. Since vitamin therapy requires, as a rule, repeated administration of small or medium doses over an extended period of time, chronic toxicity tests are indispensable for forming valid conclusions regarding the potential toxicity of a vitamin. Such tests should be conducted over the entire lifespan of the test animal and should preferably be continued over several generations (Perla, 1937; Sure, 1940).

Vitamins in general have a very low order of toxicity, the only exception to this being the vitamin D's. While some vitamins, such as riboflavin and pteroylglutamic acid produce certain lesions of the kidney, when administered in very large doses, this is due to mechanical rather than specific chemical factors and appears to be directly related to the low order of solubility of these compounds. Other toxic manifestations occasionally observed following the parenteral administration of vitamins seem largely to be of the type of an allergic reaction and can hardly be regarded as a specific toxic property of the particular vitamin.

II. Water Soluble Vitamins

1. The B Complex

Thiamine. The lethal doses on intravenous injection of thiamine hydrochloride for mice, rats, rabbits, and dogs respectively are 125, 250, 300, and 350 mg./kg. of body weight (Molitor and Sampson, 1936); subcutaneously and perorally the fatal doses were six and forty times larger. Signs of toxicity are shock, twitching of the muscles, disturbed breathing, and finally, respiratory failure. The ratio for the therapeutic dose to the minimum toxic dose in monkeys was approximately 1:15,000 (Hecht and Weese, 1937). Rabbits appeared normal after the prolonged administration of 50 mg./kg. of thiamine intravenously daily. However, Haley and Flesher (1946) have recently reported that the intravenous injection of 200–300 mg. (total dose) of thiamine usually resulted in

collapse and/or death of rabbits. Recovery occurred in animals in which the injection was terminated before respiration ceased.

Large quantities of thiamine administered to rats daily throughout life resulted in a loss of maternal instinct and disturbance of lactation in succeeding generations (Perla, 1937; Sure, 1940; Molitor, 1942; Perla, 1939; Perla and Sandberg, 1939). Perla has reported that this effect can be counteracted by the addition of small quantities of manganese to the diet.

Injection of large doses of thiamine to frogs (Demole, 1938) produces a typical curare-like paralysis of the striated muscle, which can be counteracted by prostigmine. Unna and Pick (1944) have shown that thiamine antagonizes the effect of nicotine on isolated smooth muscle; the site of this action is probably the myoneural synapses and end plates at the myoneural junction.

Toxic manifestations of thiamine administrations in humans are rare and are most likely of allergic origin. Steinberg (1938) and Mills (1941) reported that herpes zoster was observed in 3 cases following intensive thiamine therapy. Other untoward reactions were nausea, epigastric fullness, and cramps, and symptoms resembling an overdosage of thyroxine (Eisenstadt, 1942; Leitner, 1943; and Stein and Morgenstern, 1944). Williams and Spies (1938) have stated that "because of the great difference between the toxic dose, and the absence of evidence for cumulative toxicity, vitamin B_1 is a remarkably safe therapeutic agent and may be used without concern in long extended treatment." These investigators have given normal subjects 500 mg. daily for a month without any objective toxic effects; and even severely ill patients tolerated the intravenous injection of 20 times the usual dose of thiamine. Jolliffe (1941) administered thiamine to more than 3,000 patients without untoward reactions. Bicknell and Prescott (1946), during a period of 7 years, did not encounter a single case of intolerance.

Although untoward reactions following thiamine administration have been observed it should be emphasized that cases of intolerance are comparatively rare and usually occur only with parenteral administration of the vitamin. For this reason it has been suggested by the editors of *Lancet* (1947) that thiamine hydrochloride should be administered only by mouth except in severe intestinal dysfunction and acute beriberi. If parenteral administration must be used, the risk of anaphylaxis should be borne in mind and doses of 50 to 100 mg. should be given very slowly.

Successful desensitization to thiamine has been accomplished by the subcutaneous injection of graded doses of thiamine (Mitrani, 1944).

Mention should be made of the use of thiamine in the treatment of conditions other than thiamine deficiency. Thiamine is an essential

factor in the transmission of nerve impulses; its activity in this respect being due to the inhibition of choline esterase formation and thereby augmentation of the acetylcholine effect on the nerve endings (Abderhalden and Abderhalden, 1938; Jackson and Wald, 1942). Pick and Unna (1945) reported that thiamine suppressed the electrical potentials in frog brains in doses representing $\frac{1}{20}$ to $\frac{1}{30}$ of those effective on the myoneural junction; while with curare and erythroidine the doses effective in decreasing the electrical activity of the brain were slightly larger than those which blocked transmission at the myoneural junction.

Many papers have appeared on the treatment of peripheral neuritis since the initial communication of Vorhaus, *et al.* (1935). Kalaja (1941) concluded, following the treatment of 51 cases of neuritis of varying etiology, that only the symptoms due to a deficiency in thiamine respond to thiamine therapy.

Likewise, the favorable influence of thiamine on the polyneuritis and peripheral neuritis of pregnancy may be ascribed to the existence of a dietary deficiency (Bingham, 1936; Herzberg, 1941).

The role of thiamine in the treatment of heart failure was appraised in an editorial appearing in the British Medical Journal (1944). It was considered doubtful if the vitamin had any therapeutic effect on the heart except in cases of beriberi and long-established alcoholism.

Other therapeutic claims for thiamine include its effectiveness in diabetes mellitus (Vorhaus *et al.*, 1935), gout (Vorhaus *et al.*, 1935), seasickness (Holmes, 1944), radiation sickness (Whitmore, 1943), acrodynia (pink disease) (Durand *et al.*, 1939), hyperthyroidism (Williams *et al.*, 1943). However, none of these claims could be upheld upon critical examination and the beneficial effects which unquestionably resulted from thiamine administration in many of these conditions could be ascribed to the correction of a coexisting state of thiamine deficiency.

Niacin. Early experiments by Hunt and Renshaw (1929) indicated that 20 mg. of niacin hydrochloride per kilogram of body weight was nontoxic in mice and was without effect on the blood pressure of the cat. Later Unna (1939) reported the acute toxicity (LD 50) of niacin in mice and rats as from 4–5 g./kg. by the subcutaneous and 5–7 g./kg. by the oral route of administration; niacinamide was about twice as toxic. Prolonged oral administration of large amounts (up to 2 g./kg. daily) of sodium salt of niacin to rats, chickens or dogs for periods up to 2 months failed to produce toxic signs or pathological changes. The sodium salt of niacin and niacinamide were without effect upon blood pressure and respiration of the cat when administered in doses up to 1 g./kg. However, the injection of niacin (0.5% solution, pH 3.3) even in small amounts (5 to 10 mg.) frequently raised the blood pressure by 10 to 20 mm. Hg.

Since HCl at the same pH gave a like result it is justifiable to ascribe this rise in blood pressure, first reported by McCrea (1938), to the acidity of the injected solution.

Brazda and Coulson (1946) reported that niacin and trigonelline, when administered subcutaneously to rats, showed such a low order of toxicity that accurate determination was impossible. Methylation decreased the toxicity of niacinamide but was without influence on that of niacin.

The vasodilating properties of niacin were observed in humans by Sebrell and Butler (1938), Sydenstricker *et al.* (1938), Spies *et al.* (1938). The administration of niacin to both normal subjects and to pellagrins, brought about a flushing of the face and neck and a sensation of heat, tingling, and itching. Sometimes slight dizziness, headache, and nausea were observed. The symptoms were only transitory, though disturbing to the patient. These side effects were not produced by niacinamide. Oral doses of 100 to 300 mg. or 20 to 25 mg. subcutaneously caused an increase in skin temperature but not in body temperature. Sodium, ammonium and monoethanolamine salts of niacin as well as its ethyl ester produced the same reaction. The vasodilator action was reversed by adrenaline.

The administration of niacinamide in high dosage to subjects saturated with niacin did not produce the peripheral vasodilation or other vascular symptoms which followed the injection of large doses of niacin.

In addition to its effect on the arterioles of the skin, the vasodilator action of niacin is also noted in cerebral and spinal vessels (Loman *et al.*, 1941).

Calder (1947), in experiments on the action of niacin on the isolated rabbit's heart, reported that the effect on the normal organ was insignificant: in myocardial failure, however, the compound produced a marked increase in amplitude, reversal of abnormal rhythms, and at times a considerable augmentation of coronary flow. Calder is of the opinion that the disturbances of myocardial action are due to depletion of the pyridine nucleotides, and that this is counteracted by the addition of niacin to the perfusion fluid.

The nonspecific vasodilating effect of niacin has been used in the treatment of a number of diseases in which the use of a vasodilator is indicated. Niacin therapy has proved efficacious in cases of Meniere's syndrome (Atkinson, 1941), perennial vasomotor rhinitis (Williams, 1946), idiopathic or hypertensive headaches, typical migraine and headache following spinal taps (Goldzieher and Popkin, 1946) and trigeminal neuralgia (Poser, 1942; Karl *et al.*, 1945).

The vasodilating property of niacin may be regarded as respon-

sible for its efficacy in the treatment of asthma (Melton, 1943; Maisel and Somkin, 1942), angina pectoris (Neuwahl, 1942), and chronic coronary insufficiency (Uhlmann, 1944). Miscellaneous uses of niacin have been reported such as in the treatment of chilblains (Calvert, 1946), Vincent's angina (Johnson, 1945), various types of dermatoses not associated with pellagra (Schmidt, 1945) and in allergic reactions to penicillin (Service, 1946).

Mention should be made of recent studies which have shown a definite relationship between the tryptophane intake and the niacin requirement of several species. Krehl *et al.* (1945a) reported that tryptophane counteracted the growth retarding effect of corn grits in niacin low rations in the rat; a similar finding was observed in the dog (Krehl *et al.*, 1945b).

Riboflavin. Early observations (Kuhn and Boulanger, 1936) indicated that riboflavin was nontoxic when administered intraperitoneally at a level of 340 mg./kg. to mice. Demole (1938b) reported that the rat tolerated a dose of riboflavin 5000 times greater than the therapeutic dose without showing any toxic signs. Riboflavin was nontoxic to fish, frogs, mice, rabbits, cats and dogs following single or repeated doses ranging from 1 to 50 mg./kg. of body weight. Unna and Greslin (1942) reported that the LD 50 of riboflavin following intraperitoneal administration was 560 mg. per kg. Death, due to mechanical impairment of kidney function by concretions, occurred in 2 to 5 days. Oral administration of riboflavin to rats (10 mg. per kg.) and to dogs (2 g./kg.) failed to produce any untoward effects as the low solubility of riboflavin prevented its absorption from the gastrointestinal tract in amounts sufficient to produce toxic changes. Daily administration of 10 mg. of riboflavin over periods of four months to rats and 25 mg./kg. to dogs was well tolerated. Rats receiving 10 mg. daily were raised through three generations without showing pathologic signs.

Pathological changes in the urinary tract of rats were observed following the intraperitoneal administration of excessively large doses (125–500 mg./kg.) of riboflavin (Antopol, 1942). A precipitate of the vitamin was found in the kidneys and the urinary tract. The pathologic effects consisted of obstructive and inflammatory kidney lesions with resultant uremia and a calcifying nephrosis.

Riboflavin has been clinically employed in the treatment of symptoms ascribable to a deficiency of this vitamin. Since, however, under certain conditions, such as exposure to bright light and eye strain, the requirement for this vitamin is apparently greatly increased, riboflavin therapy may prove successful even when the dietary intake appears to be completely adequate (Tisdall *et al.*, 1943).

C. B. Smith (1946) has reported the improvement of patients with simple and ophthalmic migraine, when 15 mg. of riboflavin daily was given for several months.

Since the initial observation of Kinosita (1937) on the production of hepatomas in rats by *p*-dimethylaminoazobenzene, an attempt has been made to associate the state of nutrition with susceptibility to tumor formation (Nakahara *et al.*, 1939a; Nakahara *et al.*, 1939b; Kensler *et al.*, 1941). The administration of large amounts of riboflavin (10 mg., 3 times weekly) retarded the occurrence of pathologic changes in the liver produced by *p*-dimethylaminoazobenzene (Antopol and Unna, 1942). It is of interest to note that the concentration of riboflavin is lower in tumor than in normal tissue (Pollack *et al.*, 1942).

Pyridoxine. The toxicity of pyridoxine was investigated by Unna (1940) who found that the LD 50 of the hydrochloride in rats was 3.7 g./kg. following subcutaneous injection and 5.5 g./kg. on oral administration. Doses in excess of 1 g./kg. produced toxic manifestations in dogs, rabbits, and rats characterized by impairment of coordination, and tonic convulsions. The dose required to produce these signs was at least 1000 times greater than the therapeutic dose. Daily administration of 20 mg./kg. of pyridoxine to dogs and 25 mg./kg. to rats failed to produce toxic manifestations or pathological changes. Rats receiving 2.5 mg. daily were raised through three generations. Twenty milligrams per kilogram injected intravenously into cats was without effect upon blood pressure or respiration. Isolated organs (uterus and intestine) of rabbits were not influenced by pyridoxine in a concentration of 1:10,000.

The role of pyridoxine in human nutrition is unknown. This vitamin has been employed in the treatment of several types of muscular dystrophy with inconclusive results (Jolliffe, 1940; Meller, 1942; Rudesill and Weigand, 1941; and Spies *et al.*, 1940). Pyridoxine appears to give some promise in the therapy of acne vulgaris (Jolliffe *et al.*, 1942).

Recent investigations by Stoerk *et al.* (1947) and by Axelrod *et al.* (1947) indicate that pyridoxine may be involved in the development of immunity as rats deprived of this vitamin showed a striking impairment in antibody response.

The effectiveness of pyridoxine in the treatment of radiation sickness appears to be established. A large number of confirmatory reports (Reeves, 1946; Oppenheim and Lih, 1946; Wells and Popp, 1947) have appeared since the original paper by Maxfield *et al.* (1943). No rationale has been found for this action of pyridoxine since it is not known what, if any, changes occur in the blood and tissue levels during radiation therapy or whether the availability of pyridoxine is affected by such treatment.

Pyridoxine has been employed in nausea and vomiting from other causes including pregnancy and motion sickness without definite objective results. Molitor and Kuna (personal communication) investigated the effect of large parenteral doses of pyridoxine in dogs upon experimentally induced vomiting. The animals were maintained on a balanced diet, containing adequate amounts of pyridoxine and in addition were given pyridoxine up to 250 mg./kg. subcutaneously preceding, or simultaneously with, emetic doses of apomorphine. No changes were noted the frequency, severity, time of onset, and duration of vomiting. These findings indicate that pyridoxine is without effect upon the vomiting center.

Pantothenic Acid. The toxicity and pharmacology of pantothenic acid were investigated by Unna and Greslin (1940, 1941). As with the other vitamins of the B complex, the toxicity is low. The LD 50 following subcutaneous injection is 2.7 g./kg. in mice and 3.4 g./kg. in rats. The daily administration of calcium pantothenate to monkeys (1 g./monkey), dogs (50 mg./kg.) and rats (1 g./kg.) failed to produce any toxic manifestations or pathological changes. Instillation of 0.5 ml. of a 10% solution of pantothenic acid into the conjunctivae of rabbits produced no irritation. The subcutaneous injection of 1 ml. of a solution of the same concentration did not produce irritation, inflammation or abscesses in rabbits. Calcium pantothenate is likewise well tolerated in humans. Doses of 100 mg. were administered intravenously to normal persons without producing significant changes in blood pressure, pulse or respiration (Spies *et al.*, 1940).

Rats maintained on a pantothenic acid deficient diet are capable of developing heavier infections with *Trypanosoma lewisi* than normal rats (Taylor and Becker, 1948). While the omission of pantothenic acid from the diet of experimental animals produces a well defined pathological syndrome, the role of this vitamin in human nutrition has not been established.

p-Aminobenzoic Acid. *p*-Aminobenzoic acid is not a specific growth factor for the rat. It is, however, an essential metabolite for certain microorganisms. The deficiency syndrome produced in rats by the administration of certain sulfonamides in conjunction with purified diets can be negated by PABA (Black *et al.*, 1942) due possibly to stimulation of the synthesis of other vitamins by intestinal microorganisms. Briggs *et al.* (1943) have reported that PABA is not a specific growth factor for the chick but may indirectly influence the production of other vitamins.

Scott and Robbins (1942), in an investigation of the acute, oral toxicity of PABA found that the drug was better tolerated by rats than by mice or dogs. Doses in excess of 1.0 g./kg. in dogs resulted in the

death of some of the animals. Acute gastroenteritis and hemorrhages into the small intestine were observed. Acute necrosis of the liver was seen in dogs receiving 2.0 g./kg. or more. Rats tolerated doses of 1.4 g./kg. by mouth for about a month without inhibition of growth or pathological changes. No deaths occurred unless at least 6 g./kg. were fed daily for 3 successive days. Following intravenous injection of 4 g./kg., 30% of the animals died (Richards, 1942), the manifestations of toxicity being convulsions and respiratory paralysis. Young rats injected with 200–500 mg./kg. daily for 21 days grew at a slightly faster rate than did their controls. In rabbits the intravenous LD 50 was approximately 2 g./kg. High intakes of PABA (up to 2 g./kg. orally) have been tolerated by patients receiving the drug in the therapy of rickettsial infections. The chronic toxicity of PABA is low.

The blood pressure of dogs and cats was slightly raised by doses of 100 mg./kg. but this increase was dependent upon the rate of injection. Respiration was not affected. The oxygen consumption of rats was slightly but not consistently increased after intraperitoneal injection of 600 mg./kg.

PABA has a detoxicating action against massive doses of the various pentavalent arsenicals (Sandground, 1943). The protective effect was dramatically demonstrated within 24 hours following the intravenous administration of high doses of acetarsone. At a time when nearly all control rats were dead or *in extremis*, only a few animals receiving adequate amounts of PABA gave evidence of acetarsone intoxication. The drug appeared to be somewhat effective in the relief of central nervous system disturbances produced by arsenicals.

PABA in large doses is unquestionably effective in the treatment of rickettsial infections, both experimental (Snyder *et al.*, 1942; Greiff, *et al.*, 1944; Anigstein and Bader, 1945; Hamilton, 1945; Hamilton *et al.*, 1945; Snyder and Zarafonetis, 1945; Murray *et al.*, 1945) and clinical (Yeomans *et al.*, 1944; Rose *et al.*, 1945; Maroney *et al.*, 1946; P. K. Smith, 1946; Levy and Arnold, 1946; Hendricks and Peters, 1947; Ravenel, 1947a; Tierney, 1946; Tierney, 1947).

The suggested dose of PABA for the treatment of rickettsial disease is 2 g./kg./day (Ravenel, 1947b; Faust, 1946). This intake maintains a blood concentration of 30–60 mg./100 ml. Blood levels in excess of 80 mg./100 ml. may prove hazardous. Possible toxic reactions are acidosis, leukopenia, abdominal distention, and delirium. The dosage suggested by Ravenel is somewhat higher than that employed in the treatment of typhus infections by Yeomans *et al.* (1944) and Snyder *et al.* (1947).

PABA has been used by Dry (1946) and Dry *et al.* (1946), to raise the salicylate level in the blood of patients receiving salicylates in the treat-

ment of acute rheumatic fever. Rosenblum and Frazer (1947) observed that PABA per se was of value in reducing the temperature and relieving pain in rheumatic fever.

Biotin. Schmidt and Landy (1942) in a study of the pharmacological properties of biotin reported that the rapid intravenous injection of 500 γ of biotin did not influence the blood pressure, heart rate and respiration of the cat. Biotin in concentrations up to 1 part in 40,000 was without effect upon smooth muscle *in vitro;* perfusion of the frog heart *in situ* with 200 γ of biotin produced no significant alteration in amplitude, rate, or rhythm. Employing dosages as high as 5 mg. Crittenden (1948) found that biotin was devoid of specific pharmacodynamic properties; its order of toxicity was low. Mice receiving 1 mg. of biotin per os daily for a period of 60 days showed no untoward effects. Likewise, toxic signs were not observed in rats dosed orally for 10 successive days with 50 mg. of biotin.

The physiological role of biotin is unknown. Most species are capable of synthesizing biotin and a deficiency syndrome can be induced only by the incorporation of raw egg white, its active principle avidin, or one of the sulfonamides in the diet. The chick, however, is apparently unable to synthesize sufficient biotin to meet its needs.

The first experimental evidence indicating that biotin deficient chicks are less resistant to infection with *Plasmodium lophurae* than normal birds was presented by Trager (1943a,b). This observation was confirmed by Seeler *et al.* (1944b).

Susceptibility to *Trypanosoma lewisi* infection is greatly increased in biotin deficient animals (Caldwell and György, 1943). Furthermore, rats in the terminal stages of biotin deficiency were not protected from infection with *T. lewisi* by injection of hyperimmune serum in amounts that completely protected normal rats (Caldwell and György, 1947).

Biotin has been reported to have a procarcinogenic action in malnourished rats in which hepatic tumors were induced by *p*-dimethylaminoazobenzene (butter yellow). The protection afforded by casein and riboflavin when added to the ration appeared to be counteracted by biotin (du Vigueaud *et al.*, 1942). Tumor tissue contains abnormally high concentrations of biotin (West and Woglom, 1941). However, growth of spontaneous mammary cancer in mice was not influenced by the induction of a biotin deficient state (Kensler *et al.*, 1943). The feeding of large amounts of egg white did not cause the regression of neoplastic tissue in patients nor was the excretion of biotin abnormally low (Rhoads and Abels, 1943).

Pteroylglutamic Acid. The toxicology and pharmacology of pteroylglutamic acid were investigated by Harned *et al.* (1946). Due to the

low order of solubility the compound was administered as its sodium salt. As with the other members of the vitamin B complex, pteroylglutamic acid possesses a low acute and chronic toxicity and an almost complete absence of side reactions. The acute intravenous LD 50 in mg./kg. for the several species is as follows: mouse, 600 mg./kg.; rat, 500 mg./kg.; rabbit, 410 mg./kg.; guinea pig, 120 mg./kg. Thus the mouse can tolerate 5 times as much of the compound as the guinea pig. Delayed deaths were encountered in both guinea pigs and rabbits. This observation, coupled with an inverse relationship between toxicity and water intake in all species studied, suggested renal damage as a contributing factor. Pathological examination revealed that a yellow substance, apparently pteroylglutamic acid, had been precipitated in the tubules. The deposition of this compound in the kidney is similar to that observed with another sparingly soluble vitamin namely, riboflavin.

Some animals, particularly rats, died within 30 minutes after injection. Death followed a violent convulsion which was predominantly tonic.

The chronic toxicity of pteroylglutamic acid was studied in rabbits and in rats. Rabbits receiving 5 mg./kg. daily by the peritoneal route for a period of 7 weeks remained normal in all respects. When rabbits were given 50 mg./kg./day for 10 weeks a questionable retardation in growth was observed. The blood picture and general appearance were the same as with the controls. The animals at autopsy, however, showed signs of renal injury probably due to obstruction.

Some depression in growth was noted in rats given 75 mg./kg. of pteroylglutamic acid daily by the intraperitoneal route. As was the case with the rabbits, renal damage was observed at autopsy. The dosage employed in this test was far above the clinically effective range. (The prescribed daily dose in humans is 5 mg.: a 200 g. rat was given 15 mg.)

Pteroylglutamic acid did not have a significant effect upon the blood pressure or respiration of dogs, cats, or rabbits. The compound had a very low order of activity on isolated strips of rabbit intestine. The intracutaneous injection of the sodium salt produced no irritation.

Pteroylglutamic acid has been employed widely in nutrition studies following its isolation by Pfiffner *et al.* (1943) and by Stokstad (1943). The most vital of its properties is that as a hematopoietic factor in the treatment of certain macrocytic anemias in man: sprue, anemia of pregnancy, and nutritional anemia. The compound is also effective in the therapy of the dyscrasia produced in rats by the feeding of purified rations containing sulfaguanidine or succinyl sulfathiazole (Daft and Sebrell, 1943). Anemias which are produced experimentally in the

chick (Pfiffner *et al.*, 1943; Stokstad, 1943) and in the monkey (Day *et al.*, 1945) respond to pteroylglutamic acid. Numerous papers on the above subjects have appeared since the synthesis of folic acid was announced by Angier *et al.* (1945).

Although in experimental animals the efficacy of pteroylglutamic acid and of structurally related compounds is ascribable to the correction of a deficiency state, its mode of action in the treatment of certain macrocytic anemias in man is unknown. On the basis of urinary excretion figures (Wright and Welch, 1943; Johnson *et al.*, 1945) the intake of this vitamin, under ordinary circumstances, would greatly exceed the need for it. In man, pteroylglutamic acid has been effectively employed in the treatment of the macrocytic anemias of pregnancy (Moore *et al.*, 1945) and sprue (Darby and Jones, 1945). In Addisonian (pernicious) anemia the blood dyscrasia responds to folic acid therapy, but the occurrence of the degenerative changes of the spinal cord and peripheral nerves remain unaffected by the administration of even massive doses of folic acid, although a prompt response is elicited following liver therapy (Vilter *et al.*, 1946; Heinle and Welch, 1947; Meyer, 1947).

An increase in the leukocyte count was noted after the administration of folic acid to patients who developed leukopenia following roentgen-ray therapy (Watson *et al.*, 1945).

Pteroylglutamic acid was reported to stimulate the growth of sarcoma 180 transplants in female Rockland mice while pteroyldiglutamyl glutamic acid caused a regression of the tumors (Lewisohn *et al.*, 1946). This work still lacks confirmation. Farber *et al.* (1947) have presented a preliminary report dealing with clinical trials of pteroyldiglutamic acid and pteroyltriglutamic acid in man. The findings relative to tumor growth are not convincing. The adult patients, however, gave evidence of subjective improvement following the administration of the compounds. Furthermore, both preparations appeared to reduce the requirement for analgesics.

Choline. The role of choline as a dietary essential is confused by the fact that mammals must be maintained on a ration low in protein in order to produce a deficiency state ascribable to a lack of choline. Methionine can assume the function of supplying a labile methyl group in the biosynthesis of choline.

Toxicity of choline chloride has been recently studied by Hodge and Goldstein (1942), Hodge (1944) and Neuman and Hodge (1945). The LD 50 is approximately 320 mg./kg. intraperitoneally for albino mice and 6.7 gm./kg. orally for rats. An increase of toxicity with concentration was found regardless of whether the choline was given orally or

intraperitoneally. Among the toxic manifestations were respiratory paralysis and the excretion of a protoporphyrin-containing discharge from the Harderian glands.

Chicks fed diets containing 1, 2, and 4% choline showed a progressive depression in growth. Although gross pathological lesions were not observed a decrease in body fat was noted which was more marked as the level of choline was increased (Melass *et al.*, 1946).

Davis (1944, 1946) claimed to have induced a hyperchromic anemia in dogs by the oral administration of choline. However, Clarkson and Best (1947) failed to confirm Davis' findings. Livingstone and Witts (1945) were unable to produce an anemia in rabbits with choline.

It is of interest to note that Moosnick *et al.* (1945) reported the successful treatment of a case of Addisonian pernicious anemia with choline chloride. Davis and Brown (1947) employed choline chloride in the therapy of three cases of (non-Addisonian) megaloblastic anemia. Although choline chloride in itself and parenteral administration of liver were without effect, the administration of liver extract if preceded by choline was followed by excellent blood regeneration.

2. *Other Water Soluble Vitamins*

Ascorbic Acid. It is generally accepted that ascorbic acid is virtually nontoxic. Intakes in excess of the bodily needs are excreted in the urine. Demole (1934) reported that guinea pigs tolerated doses of 0.4–2.5 g. ascorbic acid/kg. daily by the oral, subcutaneous, or intravenous routes, amounts representing 500–1000 times the guinea pig's requirement for ascorbic acid. Mice receiving 0.5–1.0 g./kg. for 7 days were indistinguishable from their controls throughout the experimental period and for a fortnight thereafter. Histological examination of the tissues revealed no abnormalities. Large quantities of ascorbic acid were likewise well tolerated by rabbits, cats, and dogs. Abt and Farmer (1938) administered repeated doses of 1 to 6 g. ascorbic acid orally and intravenously to adults without evidence of toxic action. These investigators are of the opinion that vagotonic symptoms in children observed by Schade (1935) and Widenbauer (1936) are ascribable to idiosyncrasy or sensitivity to the vitamin. Maurer *et al.* (1938) gave daily doses of 1 to 3 g. ascorbic acid to 100 patients suffering from insomnia without evidence of toxic symptoms other than a slight drowsiness that disappeared 24 hours after the vitamin was discontinued.

Ascorbic acid has been reported to have a diuretic action (Abbasy, 1937; Evans, 1938). Its activity is less than that of the mercurial and xanthine diuretics, and ammonium chloride. The use of ascorbic acid

as a diuretic may be indicated when slow and progressive dehydration is desired.

The role of ascorbic acid in the formation of intracellular substance seems well established. The fact that reticulum and collagen are not formed in the scorbutic animal led to the successful trial of ascorbic acid in accelerating wound healing (Lanman and Ingalls, 1937; Lund and Crandon, 1941; Farmer, 1944; Lund, 1939; Bartlett *et al.*, 1940; Hoxworth, 1944; Bourne, 1944). Ascorbic acid apparently is also essential for the healing of fractures. The excretion of ascorbic acid falls rapidly in animals with experimental multiple fractures. The degree of healing is dependent upon maintaining an optimal level of ascorbic acid in the diet (Lauber *et al.*, 1937). The requirement for fracture repair in the guinea pig is essentially the same as in wound healing (Bourne, 1942).

Except in frank cases of scurvy it is doubtful if ascorbic acid has any effect upon tooth structure.

The reducing property of ascorbic acid may be responsible for its efficacy in the treatment of methaemoglobinemia (Deeny *et al.*, 1943; Graybiel *et al.*, 1945; Barcroft *et al.*, 1945; Lapage, 1946; Sievers and Ryon, 1945), arsphenamine poisoning (Sulzberger and Oser, 1935; Cohen, 1938; McChesney *et al.*, 1944; McChesney, 1945, Farmer *et al.*, 1940), lead-poisoning (Holmes *et al.*, 1939), benzene poisoning etc. (Libowitzky and Seyffried, 1940). Space does not permit a detailed discussion of these uses; furthermore, the therapeutic effects of ascorbic acid in these conditions, if existent, are probably nonspecific and not due to its function as a vitamin.

The high content of ascorbic acid in the adrenals led investigators to attempt to discover a functional relation between adrenocortical secretion and the production of ascorbic acid. Szent-Györgyi (1930) stated that ascorbic acid completely inhibited the formation of a pigment in all systems in which an melanoid pigment is formed through the oxidation of a phenol. Association of an ascorbic acid low state with the pigmentation of Addison's disease was a logical conclusion. A number of investigators (Morawitz, 1934; Hoff, 1936 and Rothman, 1942) reported a decrease in the pigmentation found in this disorder, following the administration of ascorbic acid. The urinary excretion of ascorbic acid was low in patients affected with Addison's disease and increased following the daily administration of 300–500 mg. of ascorbic acid (Wilkinson and Ashford, 1936). It is difficult, however, to evaluate the significance of low ascorbic acid excretion in view of the poor state of health and general nutrition of individuals with this malady.

Dugal and Therien (1947) have reported that the amount of ascorbic acid in the tissues of rats kept at low temperatures (24 and 39°F.)

increased by 80% over that of rats maintained at room temperature. Under these conditions, rats apparently synthesize more ascorbic acid to aid their body adaptation to cold. Guinea pigs were found to be able to withstand lowered temperature depending upon the amount of ascorbic acid which they received. A relation between adaptation to cold and the ascorbic acid concentration in the tissues, particularly the adrenals, was observed.

Ascorbic acid therapy has been employed with conflicting results in the treatment of varying types of infections as well as in the therapy of allergic conditions such as asthma and ragweed hay fever (Ruskin, 1945; Newbold, 1944).

Vitamin P (*Citrin*). Rusznyak and Szent-Gyorgyi (1936) reported that a flavone glucoside (citrin) which they had isolated from Hungarian red peppers and from lemon juice prolonged the life of ascorbic acid deficient guinea pigs and reduced the severity of the hemorrhages observed in scurvy. Their preparation was also effective in the treatment of the increased capillary permeability of purpura. A number of investigators, the first of whom was Zilva (1937) were unable to confirm the Hungarian workers' findings and Szent-Györgyi himself later reported his failure to repeat his original observations (Szent-Györgyi, 1938). However, more recently Zacho (1939), Rusznyak and Benko (1941), Bacharach *et al.* (1942) and Bourne (1943) who have employed a more precise method for measuring capillary fragility have demonstrated that vitamin P has this property.

Few data are available on the toxicity and pharmacology of the glucosides hesperidin and eriodictyol, which in varying proportions constitute vitamin P, citrin, and rutin. Garino (1913) administered to dogs rutin, citrin, and the glucosides, hesperidin and naringin by mouth or parenterally. None was found to be toxic. Recent investigations (Wilson *et al.*, 1947b) have demonstrated that adult rats and guinea pigs tolerated 30–50 mg./kg. of rutin by the intraperitoneal or intravenous routes of administration. Rabbits receiving 100–200 mg./kg. intravenously remained free from untoward effects.

Chronic toxicity studies were carried out with rats which had been placed on a diet containing rutin in the amount of 0.25–1%. Animals receiving such rations for long periods remained normal both in growth and in reproductive performance.

Wilson and DeEds (1940) fed naringin and hesperidin to rats for 200 days at a level of 1% in the diet without evidence of cumulative injury.

The intravenous administration of citrin caused a slight fall in blood pressure which could be ascribed to vasodilation (Armentano, 1938).

Vitamin P has been employed therapeutically in the treatment of

conditions in which increased capillary fragility has been observed, such as the purpura of rheumatic fever (Rinehart and Johnson, 1944) and retinal hemorrhages associated with hypertension (Griffith and Lindauer, 1944). Assessment of the value of rutin medication in hypertension is difficult due to the variability of capillary fragility in untreated cases. Furthermore, increased capillary fragility is not a common complication of hypertension and methods for the measurement of capillary resistance are crude.

Wilson *et al.* (1947a) have reported that rutin protects guinea pigs from histamine shock. The action, however, is indirect, as evidenced by the lack of protection when the two drugs are administered simultaneously. It is possible that this protection is due to a retardation of epinepherine destruction resulting in a slightly increased level in the blood. A recent report by Raiman *et al.* (1947) indicates that rutin may prevent anaphylaxis.

III. Fat Soluble Vitamins

1. Vitamin A

Harris and Moore (1928) and Collazo and Rodriguez (1933) described the sequence of changes occurring following overdosage with Vitamin A. Although cod liver oil and cod liver oil concentrates were employed in their studies, later investigations with pure preparations have confirmed their findings (Vedder and Rosenberg, 1938; Wolbach and Bessey, 1942; Wolbach, 1946). The administration of excess vitamin A produced roughening of the skin, rarefaction of the bones, alopecia, and profuse internal hemorrhage. Toxic doses of vitamin A accelerated, in general, those growth sequences of bone retarded by the deficiency (Wolbach, 1946).

Light *et al.* (1944) found that the hypoprothrombinemia produced by excessive vitamin A could be controlled by the simultaneous daily administration of vitamin K (2-methyl-1,4-naphthoquinone). This finding has been confirmed by Walker *et al.* (1947).

Signs of vitamin A intoxication in man described by Spiesman (1941) and Rodahl and Moore (1943) are malaise, loss of weight and appetite, headache, and giddiness.

As man relies on plants to a great extent for his source of vitamin A, mention should be made of conditions arising from an excessive ingestion of the carotenoids. This subject has been reviewed by Josephs (1944). When the carotene rises in the blood beyond a certain level (which varies with the individual) the skin becomes yellow to orange in color. This condition, the severity of which may be influenced by a coexisting patho-

logical state, is known as carotenemia. The horny layers of the skin are colored by the carotene excreted by the sebaceous glands. In contrast with jaundice, the conjunctiva, urine, and feces are not discolored.

Vitamin A concentrates have been employed extensively in the treatment of night blindness and in the therapy of follicular hyperkeratosis. However, the success encountered in such cases is ascribable to the correction of a deficiency state.

2. Vitamin D

Two steroids having vitamin D activity are of clinical interest. Vitamin D_2 (Calciferol) is the antirachitic principle produced by the irradiation of ergosterol, the main sterol of yeast. Vitamin D_3, activated 7-dehydrocholesterol, constitutes the vitamin D present in animal fats. All forms of natural and synthetic vitamin D are toxic when given in excessive dosage. Contaminants (toxisterol and tachysterol) of crude preparations may also produce untoward reactions. Pfannenstiel, as early as 1928, reported that peroral administration of large quantities of irradiated ergosterol to growing rabbits resulted in severe cachexia followed by death. Toxic manifestations of vitamin D overdosage have been reviewed by Reed *et al.* (1939).

Morgan *et al.* (1937) reported that in rats calciferol was more toxic than was the "vitamin D" of fish liver oil. Excess vitamin A appeared to decrease, but not eliminate the harmful effects of an excessive dose of irradiated ergosterol. Morgan *et al.* (1940) later found that large doses of vitamin D_3 in rats produced damage more rapidly than did vitamin D_2; however, symptomatic recovery occurred sooner from vitamin D_3 than from D_2 poisoning. Harris *et al.* (1939) found that the vitamin D of irradiated ergosterol was definitely more toxic than the vitamin D of tuna liver oil.

Young dogs are apparently more susceptible to excess vitamin D (Hendricks *et al.*, 1947) than adult animals.

Morgan *et al.* (1947) reported that young dogs were unable to tolerate a single dose of 300,000 to 500,000 units/kg. of irradiated ergosterol. Of eight 4- to 5-week old dogs employed in these studies three were dead within 2 weeks and the remainder showed signs of a high order of toxicity. Becks (1942 and Becks *et al.* 1946a) has observed that excess vitamin D has a deleterious effect upon dental and paradental structures. Even a single dose of vitamin D_2 (450,000 units administered to month-old puppies) produced osteoporosis of the mandible and degeneration of the teeth. Young dogs given excessive doses of vitamin D_2 or D_3 for a 5-month period showed profound pathological changes in dental structure. The abnormalities persisted following a recovery period of similar

duration (Becks *et al.*, 1946b). Single doses of vitamin D preparations (100,000 to 200,000 units/kg.) were employed by Wolf (1943, 1944) in the treatment of infantile rickets. These findings cast doubt upon the wisdom of employing massive doses of vitamin D in the treatment or prophylaxis of infantile rickets.

The potential danger of overdosage with vitamin D cannot be overstressed; 150,000–200,000 units as "Ertron" have been employed by Reed *et al.* (1939) in the treatment of arthritis. As pointed out by these investigators, there is a definite risk from such intakes. In fact, at least one death from intoxication with Ertron has been reported (Kaufman *et al.*, 1947).

3. *Vitamin E*

Perhaps the most striking chemical property of the tocopherols is their antioxidant activity (Olcott and Mattill, 1936). The tocopherols not only protect the fats in which they occur from oxidation, but also enhance the activity of vitamin A and carotene. The degree of synergism of the several tocopherols does not parallel their antisterility activity (Joffe and Harris, 1943; Hickman *et al.*, 1944). The relative antidystrophic potency of the several tocopherols is not known.

The pharmacological properties of synthetic α-tocopherol and its acetate were investigated by Demole (1938–39) who found neither to be toxic. Mice given orally 50 g./kg. of body weight of α-tocopherol in one day showed a weight loss of 2 to 3 g. which lasted 3 to 4 days and was ascribable to anorexia. Rats tolerated oral doses of 5 g./kg. which is 500 times larger than the effective antisterility dose in vitamin E deficient rats. Frogs, cats, rabbits, and dogs tolerated 0.2 g./kg. orally, subcutaneously, intramuscularly, or intraperitoneally.

There was no local reaction in these species except a transient hyperemia following intracutaneous injection in the ear. Oral dosage of vitamin E for 10 successive days was tolerated in a total dosage of 0.5–1 g./kg. by normal rats and 0.1 g./kg. by dogs and cats; 2.5 g./kg. subcutaneously and intraperitoneally and 25 g./kg. orally were tolerated by mice. A total dose of 4 g./kg. given orally for 2 months was well tolerated by male and female rats, as were 0.21 g. over a month by dogs and 0.25 g. by cats.

α-Tocopherol has been used clinically in the treatment of habitual abortion and of certain neuromuscular disorders. As early as 1931, Vogt-Möller in Denmark reported that wheat germ oil was effective in preventing abortions in two women with previous histories of several prematurely terminated pregnancies, an observation which he later (1939) confirmed with a larger number of cases. Since abortion may be due to many factors, therapy with the tocopherols is difficult to evaluate.

An examination of the numerous reports on the use of vitamin E in the treatment of neuromuscular disorders of varying etiologies can only lead to the conclusion that neither vitamin E alone nor in combination with other vitamins is beneficial in amyotrophic lateral sclerosis, progressive muscular atrophy or progressive muscular dystrophy.

The possible value of vitamin E in the treatment of heart disease has received recent publicity. Vogelsang and Shute (1946) and Molotchick (1947) reported the efficacy of large doses of α-tocopherol acetate (200–600 mg.) in patients with congestive heart failure. According to these investigators the compound produced a pronounced diuresis. The effect on coronary pain was attributed to either a direct action on the coronary vessels or to an alteration of the metabolism of the heart muscle.

The editors of the *J. Am. Med. Assoc.* have stated (1946) that nothing in the known pharmacological properties of vitamin E would lead one to suspect a vasodilating action, a myotonic effect, or an ability to repair damaged heart muscle in human beings.

4. Vitamin K

There are two naturally occurring forms of vitamin K, namely K_1 and K_2. Another quinone, 2-methyl-1,4-naphthoquinone (Menadione), because of its ready availability and lower cost, has been used extensively and almost interchangeably with the naturally occurring vitamin K; however, it should be pointed out that under certain conditions these compounds differ considerably quantitatively as well as qualitatively. The belief that these compounds were interchangeable was based on the like order of activity found in chicks. Subsequent investigations with other species indicate, however, that this is not necessarily the case.

The acute oral and parenteral toxicity of vitamin K_1, phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) and menadione (2-methyl-1,4-naphthoquinone) were investigated by Molitor and Robinson (1940). The oral L.D. 50 in mice was approximately 0.2 g./kg. for phthiocol and 0.5 g./kg. for menadione. No lethal effect could be produced by vitamin K_1 in doses up to 25 g./kg. The daily feeding for 30 days of 0.35 g./kg. of phthiocol and 0.5 g./kg. of menadione were toxic. A marked fall of the erythrocyte count and hemoglobin was observed in rats fed 0.1 g./kg. of phthiocol and 0.35 g./kg. of menadione while vitamin K_1 failed to produce such an effect.

Shimkin (1941) in a study of the acute and chronic toxicity in mice of six naphthoquinones with vitamin K activity, reported that in high dosage the compounds were respiratory depressants. Acute vascular congestion resulted in hemorrhagic extravasation in the renal tubules and in the liver.

Ansbacher *et al.* (1942) reported that the oral toxicities of menadione, menadiol, and its esters were approximately one-third to one-fifteenth of their subcutaneous toxicities. The manifestations of chronic damage were the result of injury to the red cells and not to the hematopoietic or other systems.

Schwarz and Ziegler (1944) observed that vitamin K lowers the blood pressure in hypertensive rats. Vitamin K in low concentrations has been reported to decrease the synthesis of acetylcholine (Torda and Wolff, 1945).

The various quinones with vitamin K activity have been employed therapeutically in the correction of hypoprothrombinemia, ascribable to a deficiency of vitamin K. While, as pointed out, menadione (2-methyl-1,4-naphthoquinone) is clinically most commonly employed and generally regarded as interchangeable with the true vitamin K, evidence is accumulating that vitamin K_1 or its oxide are more effective than menadione in counteracting the hypoprothrombinemia caused by administration of 2-(3-cyclohexyl-propyl)-3-hydroxy-1,4-naphthoquinone (Smith *et al.*, 1946) sulfaquinoxaline (Mushett and Seeler, 1947) or dicumarol (Mushett, personal communication).

Fosdick *et al.* (1942) reported that small amounts of 2-methyl-1,4-naphthoquinone prevented the production of acid when mixtures of saliva and glucose were incubated. Armstrong *et al.* (1943) found that many naphtho- and benzoquinones shared this antibacterial property. Armstrong and Knutson (1943) and Calandra *et al.* (1944) reported that the inhibitory action of vitamin K on acid formation in saliva was independent of its properties as a vitamin. 1,4-naphthoquinone had an antibacterial effect somewhat superior to 2-methyl-1,4-naphthoquinone, although the former compound does not possess more than 10% of the vitamin K potency of the latter.

The use of vitamin K preparations in the treatment of dental caries has resulted in considerable although unjustifiable publicity by the popular press.

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The Assessment of Human Nutriture

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CONTENTS

I. Introduction

1. *Definition of Terms*

Nutrition is a process or action, nutriture is a state or condition; the state results from the process. The flow of a river into a basin produces a lake: the flow is a process and the lake is a result of that process. The flow may be increased by rainfall or impeded by a dam or fouled by an effluent; the lake will be altered accordingly. Without a basin there would be no lake: without a cell there can be no nutriture. The nutrition and therefore the nutriture of different types of cells of the body differ; and the nutriture of the body as a whole, which contributes so much to the health of the body, is the integration of the nutritures of the individual cells which contribute in different degrees. Before examining the state it will be well to examine the process.

Nutrition includes the process of metabolism in cells and everything contributing to this; it is therefore partly the sum of the processes $X \xrightarrow[+\text{ catalysts}]{+O_2} Y$ in a cell. But it is more than this: it is not restricted to the processes providing energy but also includes those that build new protoplasm or that make new compounds connected with the growth or metabolism of cells. Nutrition may obviously be disturbed if there is an improper concentration in cells of X or of oxygen or of the catalysts or of Y, or it may be disturbed by poisons that interfere with the reactions. The improper concentration of X may be caused by failure to ingest, digest, or absorb the substances that are oxidized, or that give rise after ingestion to products that are oxidized, with liberation of energy: carbohydrate, fat, and protein. These substances may reasonably be termed aliments.

An aliment may be defined as a nonspecific chemical compound that is used by the body for the production of energy and may in addition be used for growth or repair. A nutrient is a specific chemical substance that cannot be synthesized by the body in sufficient amount and must therefore be provided in relatively small amount unless a closely related substance is available to the body, since it or a closely related substance is essential for the bodily processes of metabolism, growth or repair. Nutrients include compounds of certain elements (such as Fe, Mg, Zn), certain amino acids, certain fatty acids, provitamins, and vitamins. Water and oxygen are also required by the body but are neither aliments nor nutrients.

Those compounds, aliments and nutrients, are contained in food. We may define food as what is ingested by the organism and provides one or more aliments and nutrients. A foodstuff is a substance that may be treated to form food. Thus a raw potato, a foodstuff, contains the aliment starch (which is not normally oxidized in the body but gives rise to glucose which is oxidized) and (amongst others) the nutrients β-carotene, ascorbic acid, and manganese; it may be boiled to provide a food, or it may be combined with another human foodstuff—a part of a cow—to form the food called cottage pie. A meal is the sum of one or more foods ingested at one period, and a diet is the sum of the daily or weekly meals considered especially in relation to their quality and effects. A dietary is a prescribed or adopted course of diet.

Our symbiotic microorganisms in the gut give rise to certain problems of definition. If urea is converted by bacteria in the gut into protein or polypeptides that are adsorbed, with or without preliminary digestion, are we to regard urea as an aliment? Similar problems may arise with cellulose and with the synthesis of nutrients by microorganisms. In all cases the precursors must be regarded as aliments or nutrients, and the microorganisms as contributing to true symbiosis. We must regard the activities of these microorganisms as a normal part of our economy, just as is the activity of pepsin. But it will usually be convenient, without further qualification, to regard the difference between nutrients as the difference between, say, manganese, thiamine, and calciferol, rather than between pyridoxal and pyridoxamine.

The nutrition of the cell depends then upon an adequate concentration of aliments within the cell or even within certain of its compartments; and this involves adequate ingestion or production in the gut, digestion, absorption, and then, perhaps after other processes, transport in the blood, diffusion into extracellular fluid and passage into the cell. The cell's nutrition also depends upon an adequate supply within the cell of certain nutrients, water, and oxygen, and upon the removal of waste products.

Further, if the chemical processes within the cell upon which its nutrition depends are interfered with by anything, whether by a chemical substance such as arsenic or by a physical condition such as a rise in temperature, impaired nutrition may result. This article is mainly concerned with malnutrition, and its result, malnutriture, caused by absorption from the gut of aliments and nutrients in inadequate amounts.

2. Physical State, Health, and Nutriture

It is proper here to distinguish between these three states.

Physical state is the condition of the body with respect to health (including nutriture) and structure. Physical state is said to be perfect when the body is in full health and has no structural defects that affect health. Such defects include deformities or malformations, congenital or acquired, including the scars of past malnutriture or other disease: examples are carious teeth, rachitic deformities, impaired vision, mitral stenosis.

Health, which is included in physical state, implies mental and physical wholeness; but it takes no account of structural defects caused by past and now inactive disorders unless general function is seriously impaired. An adult with only one leg, or with carious teeth or a cleft-palate may be in full health. But if general function becomes seriously impaired even by an inactive pathological process, full health cannot be said to exist: a severe mitral stenosis in an adult, caused by an acute rheumatic infection in childhood, certainly impairs health and may cause death; rats on a diet free of fluorine die of starvation because severe caries prevents chewing (McClendon, 1944).

Nutriture has already been defined and clearly plays an important, although not exclusive, part in maintaining health. It is possible, for instance, to have normal general nutriture combined with an acute infection such as appendicitis; though general nutriture may be normal, health and therefore also physical state may be impaired.

The following diagram may help to make the relationship clear (Sinclair, 1948b),

- Physical state
  - Health
    - Nutriture
    - Nonnutritional disease
  - Structural defects not affecting health
    - Congenital
    - Acquired by
      - Disease
        - Past malnutriture
        - Nonnutritional disease
      - Accident

3. *Properties of Nutriture*

We must consider three important properties of nutriture: quality, degree, and duration.

Quality includes specificity. This, however, is not absolute, but arises from the fact that deficiency of a particular nutrient tends to produce a particular pattern thereby permitting clinical diagnosis. The pattern, however, will depend upon all the other properties of nutriture. There is no reason to doubt that deficiencies of two separate nutrients can independently produce a single clinical sign: angular stomatitis can apparently arise from deficiency of iron or of riboflavin. And this need arouse no surprise when it is appreciated that such a sign can be caused by a break in a metabolic chain, and this break can occur even at the same link in the chain through deficiency of either of two nutrients. In this sense specificity is not absolute. Moreoever, it is wrong to suppose that the lesions produced by combined deficiencies are the sum of the lesions of the individual deficiencies. In rats deficiency of potassium or of thiamine causes myocardial necrosis, but deficiency of these two nutrients together is accompanied by no pathological change in the heart (Follis, 1942). Deficiency of niacin in man causes a scarlet tongue and deficiency of riboflavin causes a magenta tongue, but the tongue of the combined deficiencies will not have the tint that arises by mixing these two colors; indeed, when a pellagrin is treated with niacin without other dietary change his tongue may turn from bright red to magenta and angular stomatitis may appear, both events indicating a concomitant deficiency of riboflavin.

Quality expresses these kinds or types of deficiencies. In the case last mentioned, the quality of the malnutriture would be twofold: deficiencies of niacin and of riboflavin. Quality takes account of nutriture respecting the various nutrients and aliments, singly and in combination. If there are n different aliments and nutrients, and the different possible combinations of them as sufficient or deficient produce different qualities of malnutriture, the number of such qualities is 2^n. For man, n is probably at least 50, and therefore in theory there could be at least 10^{15} qualities. But the quality of nutriture is not only altered by deficiencies or combinations of deficiencies: poisoning with trivalent arsenic causes malnutriture of particular cells. Therefore the number of possible qualities becomes almost infinite, and it is necessary and indeed customary to consider certain typical ones. The types chosen, however, are often not as specific as is stated. It frequently happens that inanition accompanies deficiency of a specific nutrient, and therefore to the specific signs of this deficiency may be inadvertently added the

signs of deficiency of aliments: diminished adipose and lymphoid tissue, atrophy of the genital organs, diminished osteogenesis, and others.

Though the distinction is artificial, it is sometimes convenient to speak of general nutriture (of the whole body or of certain cells of the body), or of nutriture regarding specific nutrients. When we know more of the interplay of aliments and nutrients, and therefore of the quality of nutriture, the distinction will be needed less. The quality of nutriture pertaining to aliments alone is reasonably called nourishment, undernourishment being a particular type of malnutriture. The word nutriment will be used to include aliments, nutrients, water and oxygen.

4. *Degrees of Nutriture*

Obviously there can be degrees of nutriture of individual cells or groups of cells, or of the body as a whole. It is desirable to define these degrees, and an interesting attempt to do so has been made by Dann and Darby (1945). I had also attempted this as follows and prefer to retain my classification for reasons that will be stated:

(i) *Excess Nutriture.* The state in which impaired function or defective structure results from an excessive supply to certain cells of the body of one or more aliments or nutrients.
(ii) *Normal Nutriture.* The state in which function and structure are unimpaired by nutrition, and the reserves are adequate for the usual needs of the body.
(iii) *Poor Nutriture.* The state in which function and structure are unimpaired by nutrition, but the reserves are inadequate for the usual needs of the body.
(iv) *Latent Malnutriture.* The state in which function or structure is impaired by nutrition, but disease exists only *in posse* or in an undeveloped form.
(v) *Clinical Malnutriture.* The state in which impaired function or defective structure, produced by nutrition, causes definite disease, even though this may not be capable of diagnosis by a physical examination.

Death occurs at either end of the scale, from surfeit or deficiency.

Excessive ingestion of carbohydrate causes obesity; of fat, ketosis; of fluorine, brittle teeth; of iron, rickets; and hypervitaminoses have been described of various vitamins, such as A, D and thiamine. The degree called "saturation," used by Dann and Darby, should be avoided in a classification; there is no evidence that saturation is desirable and there is definite evidence that in some cases it is undesirable, as for instance in the examples just mentioned of the results of excessive ingestion; in

some cases, for example with carbohydrate or fat or vitamin A, it is not clear what saturation means. Dann and Darby include under "potential deficiency disease" the state in which a biochemical test reveals a decreased "ability of the organism to withstand a stress without deviation from its usual physiological course." The amount of ascorbic acid in 100 ml. of the plasma of a person saturated with this nutrient is about 1.7 mg.; if estimated in a person and found to be only 1.0 mg. it might reasonably be supposed that the person had decreased ability to withstand the stress of a scorbutic diet, yet one would hesitate to say that the person had potential scurvy. It is less unsatisfactory to define reserves in terms of the usual needs of the body in its usual environment: those who habitually live in arctic climates or who are accustomed to undergo dietary privations at certain seasons should have larger reserves than are needed by others; normal nutriture implies reserves that are adequate for the customary or current needs but are not necessarily maximal.

Even though function is unimpaired, the reserves of nutriment may be insufficient for the usual needs of the body. This state may be detectable biochemically by analysis of body fluids or tissues; or it may be detectable by subjecting the body to an insult that is not greater than the stresses it encounters in its usual activities and finding that function is now impaired whereas with increased reserves it would not be. This degree merges into the next in which structure or function is impaired in the absence of an added insult, but the person probably has no definite symptoms, and no clinical signs may be detected without the use of special tests of function or structure. This state corresponds to the "Latent deficiency disease" of Dann and Darby.

When there is definite deficiency disease, with marked structural changes in the tissues, the last state is reached: clinical malnutriture. Even in severe cases of this state, it may be necessary to establish the diagnosis by special tests, such as biochemical estimations, or by therapeutic trial.

It will be appreciated that the boundaries between the five degrees are arbitrary, and one degree merges into the next.

5. *Duration of Nutriture*

The last property of nutriture, duration, is concerned with the time during which the state has existed.

Some people (e.g., Kruse, 1943) fall into the error of classifying duration in terms of acuteness and chronicity. These are not determined by the duration of a particular degree of nutriture, but by the first differential coefficient of degree with respect to time. An acute or chronic process is one whose degree is changing rapidly or slowly. Except in the

rare case of a process that is alternately and rapidly advancing and receding in degree, an acute process cannot in general be of long duration because, if advancing, it will soon lead to death. If the degree of nutriture is plotted against time (Fig. 1), the slope of a line will determine rate of change of degree and therefore acuteness or chronicity. The evolution of the process or resulting state can be defined by any continuous line provided two rules are observed: first, the process or state cannot regress with respect to time; and secondly, we shall not allow the state to pass from death to one of the other degrees (a point probably overlooked by Kruse when he states that changes, whether acute or chronic, are reversible provided therapy is sufficient in amount and duration).

If rate of change (di/dt) of degree (i) with respect to duration (t) be plotted (Fig. 2), the position on the graph will determine the acuteness or chronicity of the process at a particular time.

It appears from clinical observation and from the experimental work on pigeons of Swank (Swank, 1940; Swank and Bessey, 1941; Prados and Swank, 1942), that acute deficiency of thiamine causes an accumulation of pyruvate in the lower parts of the brain and there follow capillary dilatation and then hemorrhages such as are found in Wernicke's encephalopathy; whereas chronic deficiency causes peripheral sensorimotor neuritis because the metabolism of the neurones is deranged, and if sufficiently prolonged permanent loss of function may result. Here we have a simple example of how rate of change and duration of malnutrition lead to two different states of malnutriture.

The mention of chronic lesions, and in particular of irreversible lesions, raises a difficulty. It is obvious that it is possible to have optimal nutrition coexistent with definite malnutriture: after severe deficiency has developed, therapy may restore all the metabolic processes to normal but if the processes of repair are continuing the clinical signs, though reversible, may still exist and therefore malnutriture may still be apparent. What then of permanent paralysis caused by athiaminosis or of permanent rachitic deformities? Since it is wrong to regard these as necessarily due to an existing malnutrition and confusing to regard them as manifestations of present malnutriture, it is convenient to restrict the latter term to states that are reversible by appropriate nutrition.

Kruse (1942a, 1943) has developed "a concept of the deficiency states" which may briefly be considered here. He appears to generalize about all deficiency states from his views concerning macroscopic and microscopic lesions in deficiencies of four vitamins: vitamin A, niacin, riboflavin, and ascorbic acid; the fact that these views are not acceptable is immaterial in this context. Kruse ascribes certain properties to the pathologic process in the tissue in deficiency disease, namely, velocity,

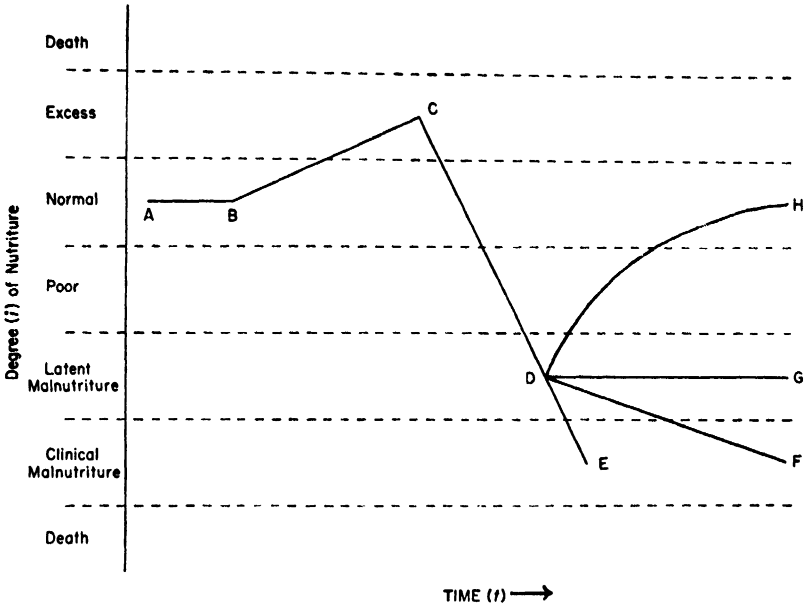
Death
Excess
Normal
Poor
Latent Malnutriture
Clinical Malnutriture
Death
Degree (i) of Nutriture
A
B
C
D
E
F
G
H
TIME (t)

Fig. 1.

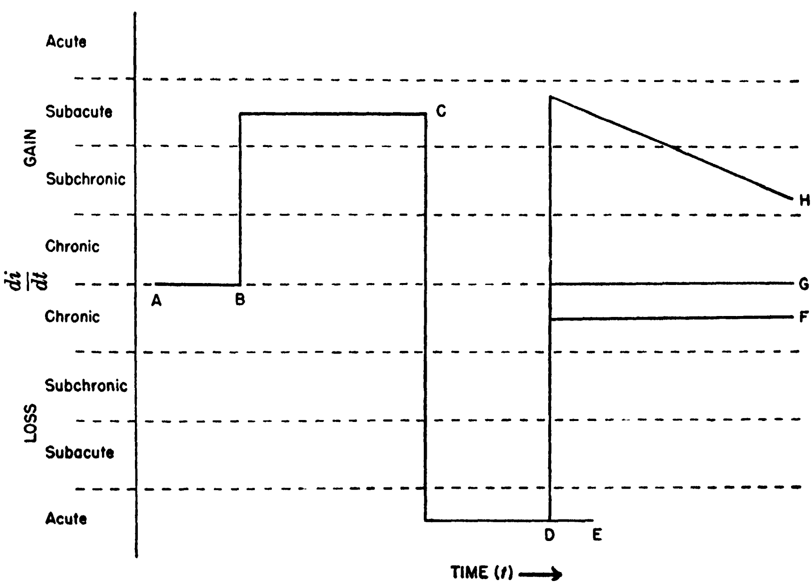
Acute
Subacute
Subchronic
Chronic
Chronic
Subchronic
Subacute
Acute
GAIN
LOSS
$\frac{di}{dt}$
A
B
C
D
E
F
G
H
TIME (t)

Fig. 2.

intensity, and duration. Velocity (also called "rate" and "form") gives rise to acute or chronic processes, "intensity" (also called "degree") to mild or severe processes, and "duration" is time. Velocity is determined by the causal force which is a function of degree and rate. It is at once apparent therefore that velocity cannot be synonymous with rate (or form) if it includes both rate and intensity. Further, since (as Kruse shows in a diagram) the essential difference between acuteness and chronicity is regarded as being in one dimension only, namely in time (or duration), the only dimension of velocity appears to be in time.

We must be clear in any such discussion whether we are considering the degree of deficiency of a nutrient in the diet or in the cells of the tissue, or the resulting degree of alteration in the process of nutrition in the cells, or the changed degree of nutriture produced by this alteration (i.e., the different state caused by the altered process). Each, of course, tends to follow the preceding one with inevitable temporal separation. With minor modifications the discussion in the earlier sections is applicable to the deficiency in the cells, to the process in the cells or to the resulting state. An acute deficiency in the cells, that is, a rapid change in concentration of nutriment in those cells, will produce a rapid change in the cellular metabolic processes and a state that may arise quickly and may be different in type from one that is caused by a slower alteration in the process. Conversely a slow change in concentration of nutriment in certain cells, which can be caused by an acute dietary deficiency when the relevant stores of the body are abundant, is likely to produce changes slowly in the metabolic processes with a nutriture of a different pattern.

Before further discussion it would be well to consider the general method of development of malnutriture.

6. Development of Malnutriture

It has been stated elsewhere (Sinclair, 1944) that malnutriture usually develops by stages which correspond to the above-mentioned degrees: first there is inadequate ingestion perhaps detectable by dietary methods and accompanied by decreased excretion, then decreased bodily reserves perhaps detectable by biochemical methods, then impaired function, then structural derangement that may be revealed at first by microscopical and later by gross anatomical changes.

These stages in development may be illustrated by an actual case, the later stages being added from experience with induced deficiency; their course may be followed on Figs. 1 and 2. Mary C., aged 24 years, was referred to me as a case of Eales' disease (angiopathia retinalis juvenalis) with the suggestion that it might be due to deficiency of ascorbic acid. Her history was that she had recently come from Ireland, and suddenly

developed misty vision in the right eye. On examination she was found to have haemorrhages into the posterior chamber of the right eye; she had marked xerosis of the skin of her arms of recent origin and her teeth had lately begun to break. Hypervitaminosis A was considered, but she admitted to taking no medicines. However, the dietist in the course of a diet history elicited the information that since coming from Ireland she had consumed daily over 60,000 I.U. of vitamin A in cod liver oil because she was told this would protect her against the English winter. She was in degree (i)—excess nutriture with impaired visual function, and impaired structure of capillaries, teeth, and skin, caused by hypervitaminosis A. In Ireland she had been travelling along the line AB, in England BC. Let us assume she stopped her self-medication (at point C) and, fearing vitamin A, excluded this and its precursors from her diet. She would pass through a transitory stage of normal nutriture, even though she retained some of the stigmata of the previous disease such as broken teeth; her plasma might contain 100 I.U. of vitamin A and 150 γ of carotenoids, and the rod threshold of her eye when fully dark-adapted might be 1.7 log μmL. Next the carotenoids in plasma would fall quickly to about 20; the plasma vitamin A might fall slightly and the rod threshold rise slightly but not significantly. She would now be in the degree of poor nutriture, detectable only by biochemical methods. The plasma carotenoids would remain approximately constant, the vitamin A would fall perhaps to 35 I.U., and the rod threshold would rise perhaps to 2.3 log μmL.; this stage of partial night blindness and low plasma values indicates the fourth degree, latent malnutriture (point D): function is impaired but there is no definite deficiency disease. This, however, finally develops (point E). In the stage of clinical malnutriture the plasma vitamin A is very low, there is markedly deficient rod function, and clinical signs (such as xerosis conjunctivae, Bitot's spots, xeroderma, and perhaps follicular keratosis) may be present.

In this hypothetical example, deprivation is obviously acute. Let us assume that when Mary C. had reached point D (latent malnutriture) she slightly modified her diet to include some vitamin A, but less than her daily requirement; the deterioration would be slowed, becoming chronic (DF) rather than acute (DE). If she took just that amount of vitamin A and its precursors that equalled the daily utilization, the process might remain at a steady state with neither deterioration nor remission (DG); this is of course maximum chronicity since the rate of change is zero. If she improves her diet sufficiently, improvement in degree will occur (DH).

This process of improvement is not simple. The poor dark adaptation is in some cases cured in a matter of hours, a very acute response.

But if clinical changes had occurred, some might have responded slowly, some very slowly, and others not at all. As we have already said, if a particular lesion is reversible, the defect of nutriture cannot be said to have been completely remedied until it has disappeared, even though nutrition be normal.

7. *Methods of Assessing Nutriture*

We start from the premise that the full deficiency state, whether acute or chronic in origin, whether mild or severe in degree, usually develops in the four stages already mentioned: inadequate ingestion, decreased bodily reserves, impaired function, anatomical lesions. It follows that methods should be capable of detecting the abnormalities at these four stages: dietary, chemical, functional, and clinical methods (the word clinical being used in a restricted sense). In addition, we have somatometric methods, since before adulthood the growth of the body is profoundly affected by nutrition; and after adolescence changes, particularly in weight, occur as a result of nutrition. In subsequent sections it will be convenient to consider these five methods separately.

8. *The Concept of Normality*

Before considering the different methods of assessing nutriture it is necessary briefly to consider our standard for comparison. We have used the term "normal nutriture"; we have conceived of function being "impaired," of bodily needs being "usual," and of "definite disease" existing. Obviously the concept of disease and the diagnosis of disease imply that there is a state of nondisease, and this is customarily called the "normal" state of health.

There are three distinct concepts of normality, whether concerned with structure, function, or the chemical composition of the human body or its parts. The first is the concept of perfection: a "normal" person is one who is perfect according to some arbitrary set of standards. The view might merit careful consideration were it not that what is ideal or optimum in one set of circumstances may be a handicap in another. If deficiency of thiamine increases the resistance of the body to an attack of poliomyelitis, the ideal state when exposed to infection by the virus is quite different from the ideal state when partaking of a diet relatively high in carbohydrate. The ideal physical state for the marathon runner is obviously different from that of the German town dweller compelled to combat the rigors of another winter. Ideal ascorbic acid nutriture has been assumed by some to be the state of saturation in which the vitamin is spilled in the urine. The concept of perfection does not satisfy.

Secondly, according to the concept of pathology a person is "normal" who is free from the signs or symptoms of active disease; mild imperfections of the body are regarded as normal. According to Ryle (1947), the realm of the pathological includes structural or functional variants that are so pronounced as themselves "to occasion indisposition or inconvenience," or that have been "further increased or actually induced by disadvantageous stimuli or habit." Though perhaps satisfactory for the clinician, who as this word implies tends to be concerned with gross departures from normality found in patients in bed, the concept of pathology is unsatisfactory for the physiologist, as Ivy (1944) has shown in his very interesting discussion of normality.

Thirdly, the statistical concept of normality varies, as Ivy points out, depending on where it arbitrarily draws a dividing line between normal and abnormal. If a variate in a homogeneous population is measured, and if the variations are described by a Gaussian distribution curve with standard deviation σ, then about 68% of the population will fall within $\pm\sigma$ of the mean and about 95% within $\pm 2\sigma$. One or other of these limits is usually taken as arbitrarily describing the limits of normality. This sharp arbitrary distinction between normal and abnormal can be avoided by merely stating in statistical terms the extent to which any individual in the population deviates from the mean of the population in respect of the character measured. Though for many purposes the statistical concept is essential, it does not satisfy the customary meaning of normal for the physiologist: it would class dental caries as being normal in an adult population, and would probably class acne vulgaris as being normal in an adolescent population.

Ivy (1944) therefore states that "the body is in a physiologically abnormal state when its relatively steady state is disturbed." It would appear however that by this definition changes at puberty and the climacteric, as well as sleep or waking, would have to be regarded as abnormal physiologically. Ryle (1947) appears at times to adopt the concept of pathology (see above), at times the arbitrary statistical concept ("When . . . the thyroid gland is invisible in upwards of 93% of cases, it seems reasonable to regard invisibility as 'normal'"); although he writes of "the normal range" without defining it, he concludes that life "admits no constant and no norm." The physiologist, following Bernard, recognizes constants: the possession, for instance, by members of a human population of two eyes, biconcave erythrocytes, and (after infancy) an upright walk. If there is a "normal range," there must be a "norm."

I propose, following Ivy (1944), to adopt both a physiological and a statistical definition, in the latter case signifying "statistical normal" and

implying by this the limits not outside $\pm\sigma$ of the mean of a variate in a population of stated characteristics. Unless thus specified, normality will be defined in physiological terms: the condition of the body, or of some part or function of the body, may be said to be normal when it allows the usual functions to be adequately performed in the usual environment. The terms "usual" and "adequately" imply, of course, arbitrariness in the definition; "usual" means customary or current. Sometimes it will be convenient to use the statistical definition; the possession by an adult man of six digits on each hand or 50,000 leucocytes/μl. of blood might allow the usual functions to be adequately performed in the usual environment, but would be statistically abnormal.

II. Dietary Assessment

Except in rare instances, dietary methods cannot alone be used to assess nutriture: in general their limitations are too great. Obviously, however, there are levels of intake below which malnutriture must occur in anyone: whoever eats no food for a month will undoubtedly suffer from deficiency of aliments. Dietary methods will therefore be mentioned only briefly.

Food consumption is estimated at various levels of distribution. Sometimes the "food moving into civilian consumption" is estimated by adding imported food and home-produced food, subtracting exported food and food used by noncivilians, and dividing the result by the civilian population. This crude method need not concern us. Food consumption may be estimated as distributed wholesale; or as purchased retail by the housewife; or as eaten after allowing for edible waste (the net result of losses and gains in cooking and of serving-waste) and inedible waste (which is preparation- and plate-waste), or as assimilated after allowing for further losses through excretion in urine and feces, and additions through aliments and nutrients synthesized or made available by bacteria in the gut. Obviously one must be clear at what stage food consumption is being assessed: there is confusion on this point even in Orr's classical study, "Food, Health and Income" (1936). Waste of various types must often be estimated, usually with considerable margins of error. For instance, waste that is allowed in most tables calculated on an "as purchased" basis is far too generous by wartime experience in England and especially by present experience on the continent of Europe.

There are five methods that are used to compute food consumption:

1. *National studies* in which the food resources of a country are computed and expressed *per capita* or by the use of some scale of man-equivalents.

2. *Institutional studies* in which the total food consumed by a homogeneous social group is computed.
3. *Budgetary studies* or family living studies, in which the amount of money actually spent, or calculated to be available for spending, on food is assessed and compared with the supposed cost of the cheapest adequate diet.
4. *Family studies* in which, usually, inventories of household stores at the beginning and end of a week are made, and food entering the house during this period is recorded; from this information, by various devices, the food consumed by the family is assessed.
5. *Individual studies* whereby the food consumed by a single person is assessed by one of three methods: first, by a detailed dietary history; secondly, by a questionnaire; or thirdly by weighing or otherwise measuring the meals consumed. This last method, which is the most accurate, has been developed in particular by Widdowson (Widdowson, 1936; Widdowson and McCance, 1936; 1942; McCance *et al.*, 1938; Widdowson and Alington, 1941; Widdowson, 1947).

The errors and advantages of these different methods cannot be discussed here.

But even if the precise amounts of food consumed were known, the problem of translating these into nutrients arises. The errors, which have been investigated by McHenry (Patterson and McHenry, 1941; Young and McHenry, 1942, 1943; Semmons and McHenry, 1944) and by Widdowson and McCance (1943), may be very large for sundry reasons which include variations in nutrient content of different types of a foodstuff (such as different sorts of apple or of jam) and incorrect allowances for losses in cooking; in the North Carolina survey, no allowance was made for ascorbic acid present in cooked food and therefore "an average daily intake of 23 mg. vitamin C, which is about one-half the minimum normal standard" was encountered (Milam, 1942); when the presence of ascorbic acid in cooked food was appreciated, "the resultant mean intake would seem to approach the recommended dietary allowance" (Milam and Darby, 1945).

The main interest in the assessment of the dietary intake of aliments and nutrients lies in estimating from the assessed intake the amount of these available in the body and comparing the available aliments and nutrients with the individual's supposed requirement for normal nutriture. The difficulties in assessing availability and requirements have been discussed by many authors (e.g., Pett, 1945), and some have been briefly mentioned elsewhere (Sinclair, 1947b) with particular reference

to calories. Perhaps dietary niacin may be cited as an example here. The National Research Council (1945) recommends 15 mg. daily of niacin as a dietary allowance for a moderately active man, this figure being based on the belief that the requirement of niacin is probably about ten times that of thiamine (for the requirement of which our knowledge is far from precise). There is evidence that niacin may be synthesized by bacteria in the gut and then absorbed (Benesch, 1945), that tryptophan is a precursor of niacin (Perlzweig *et al.*, 1947), and that a toxic factor may be involved in the production of pellagra (Woolley, 1946; Kodicek *et al.*, 1946). It is therefore impossible to compute the dietary requirement of niacin, and the dietary allowance will be a matter more of fancy than of fact. Thiamine is not only synthesized in the gut but also gets destroyed there: about half the thiamine in a meal may be destroyed if, for instance, raw clams are consumed at the same time (Melnick *et al.*, 1945). It therefore becomes extremely difficult to state how much of certain nutrients actually becomes available in the body, and what is the requirement for normal nutriture either in the food ingested or in the food absorbed from the gut. Also, physiological adaptation can occur and alter the requirement of nutrients (Mitchell, 1944). Finally, the biological action of certain aliments or nutrients in certain combinations is known to be different from that obtaining in other combinations; and the efficiency of utilization of ingested nutrients or aliments may differ under different circumstances, as shown, for instance, by Sure and Dichek (1941).

Although dietary methods cannot usually be used alone to assess nutriture, a dietary history is an essential part of such an assessment and must be included even in rapid nutritional surveys. To such surveys family dietary studies are a valuable addition, although it is not usually practicable to conduct them on more than a small sample of the subjects included. The purpose of including them is not to assess nutriture from their results, but to aid in interpreting the cause of and suggesting a remedy for any malnutriture found. Weighing or estimating by homely measures the food consumed by individuals is seldom practicable in such rapid surveys.

III. Chemical Assessment

1. *Introduction*

In the chemical assessment of nutriture, substances may be estimated in tissues, blood, urine, or other fluids (such as milk, c.s.f., or sweat). The substances usually estimated fall into three groups: first, nutrients or aliments in the resting condition or after a test dose; secondly, normal

chemical constituents that are affected by nutrients or aliments; thirdly, metabolic products whether estimated in the fasting condition or after some treatment. Measurements of erythrocrit or leucocrit, or of the number or size of erythrocytes, or of the number and type of leucocytes, are not chemical but it is convenient to include them here.

The aliments and nutrients that are estimated include protein (or different classes of proteins), amino acids such as cystine, glucose, vitamin A, carotenoids, ascorbic acid, thiamine, cocarboxylase, niacin, riboflavin, vitamin E, calcium, and phosphorus. Compounds affected by nutrients include hemoglobin, alkaline phosphatase, and prothrombase; metabolic products include pyruvate. With some compounds, such as hemoglobin or pyruvate, variations can be caused by more than one nutrient. The commoner methods of estimation we use have been summarized elsewhere (Sinclair, 1942, 1947a); other accounts include Youmans and Patton (1942), Mickelsen (1943), and papers by R. E. Johnson and Bessey. The very important alterations in blood composition caused by certain anticoagulants, by stasis during venepuncture, and by storing the blood cannot be discussed here; nor can the effect of alterations in blood volume as caused for instance by posture, activity, or dehydration.

2. Composition of Tissues

The obvious but important general principle may be stated that the composition of tissues alters before structural changes occur. It is surprising therefore that so little attention has been paid to the analysis of tissues. Muscle or liver biopsies are not easy. Hair and nails (in men or in adequately shod women) are readily collected and might prove quite useful for certain purposes: in our surveys during the famine in the Netherlands we collected samples of hair to try to obtain information about the previous protein nutriture when the hair was being formed.

The easiest and most satisfactory living tissue to obtain in nutritional surveys is leucocytes. They may be collected by the method of Butler and Cushman (1940) and then weighed, or spun to the graduated tip of a centrifuge tube after laking the erythrocytes; in Bessey's microtechnique the quantity of leucocytes present in solution is judged by the phosphorus content (Bessey *et al.*, 1947), but the soundness of this technique has not been established. The leucocytes are particularly valuable for the water-soluble vitamins. In the case of ascorbic acid, the excellent study by Crandon showed (Crandon *et al.*, 1940) that on a diet that produced scurvy the plasma ascorbic acid reached zero after 41 days, and the leucocyte ascorbic acid after twice this time and only shortly before clinical signs developed; the ascorbic acid in whole blood fell more rapidly than the leucocyte values but less rapidly than the plasma ascorbic acid.

Vitamins of the B complex are also conveniently and profitably estimated in leucocytes, and the advantages of this must now be discussed.

3. Composition of Blood

Under this heading we may include estimations upon whole blood or upon plasma or serum. The leucocytes, which may be regarded as "living" cells whereas the erythrocytes may be regarded as "dead," normally constitute such a small proportion of whole blood that their contribution to whole blood analyses is slight. Even when the plasma value is low and the leucocyte value high, the leucocytes may contain only a quarter of the total ascorbic acid in blood although they may contain 150 times the amount in an equal weight of plasma; in a person saturated with ascorbic acid, the plasma may contribute three-quarters, the erythrocytes a fifth, and the leucocytes only a twentieth of the ascorbic acid in whole blood.

Compounds in plasma behave in two strikingly different ways. Some, such as carotenoids or ascorbic acid, tend to reflect the immediate past intake: on a diet low in the particular nutrient, the plasma concentration drops rapidly; and when the nutrient is added to the diet there is a quick rise in plasma. Others, such as niacin and riboflavin, and usually protein, remain at concentrations that are within the average or statistically normal range even though clinical malnutriture, such as pellagra or ariboflavinosis, may be present. That the values are within the statistically normal range does not mean that no fall has occurred when there has been diminished intake of the nutrient. We believe on the present scant evidence that when a person subsists on a diet low in protein the serum protein concentration may fall slightly and then remain within the statistically normal range, protein meanwhile being taken from the tissues; later, depletion of the tissues may lead to a fairly rapid fall. On the other hand it is probable that a diet deficient in particular amino acids, such as lysine, may cause a rapid fall in serum protein without the initial plateau. A diet very low in animal protein is certainly compatible with a serum protein value within the statistically normal range; this was shown in one case by Abelin and Rhyn (1942), and the Oxford Nutrition Survey observed it repeatedly in the Netherlands and Germany (Sinclair, 1948a). But we have sometimes found unusually high protein values in groups of malnourished persons, such as Dutch children evacuated to England during the famine in the Netherlands or returned war prisoners; the cause of these high values, which is not dehydration, is being investigated. A single estimation of serum protein in an individual, therefore, gives little information if it falls within the statistically normal range; a value of, say, 6.3 g./100 ml. may be the level found in a particular person

however rich his diet be in protein; or it may be the level found in a person who has subsisted on a grossly deficient diet and who previously had a higher level. On the other hand significant differences between the means of population groups that are fairly homogeneous, and similar except for diet, are important; and a slight fall in a longitudinal study on an individual is valuable provided the nondietary factors that affect protein values (which have been carefully investigated by Lange (1946)) are remembered.

A similar example with thiamine may be given from a joint experiment in which 11 volunteers were placed upon a diet very low in thiamine, an equal control group receiving a daily supplement of the vitamin. After 86 days, the control subjects had a mean value of 6.50 γ/100 ml. of blood, with a standard deviation of 0.87, while the levels in the experimental subjects had a mean value of 3.95, with a standard deviation of 0.61. The ranges of values were then 5.5 to 8.0 in the control group and 3.0 to 5.0 in the experimental group, and were therefore just distinct. The subject whose value was 5 0 had at the start a value of 7.5 and after therapy at the conclusion of the experiment 7.0: had he been found at random in the population one could not have inferred from a single blood analysis that he was on a diet deficient in thiamine, but a longitudinal study of the values for thiamine in blood allowed the 22 subjects to be sorted with complete correctness into the control and experimental groups at a time when an attempt to do so on the basis of longitudinal clinical studies placed the subjects at random in the two groups. Similar results were obtained with independent estimations of cocarboxylase in blood.

We need much more information about blood levels, for example why a certain individual has a serum protein concentration of 6.5 g./100 ml. whereas another may have one of 8.5, both being on excellent diets. Somatotype is probably one important factor, but there are many others. We do not know why hyperthermia causes a rapid depletion of vitamin A in the liver with a sudden fall in serum vitamin A, whereas a diet almost free of vitamin A and carotenoids may cause no fall in serum vitamin A for months, and yet on other occasions may cause a fall in days (see below under Section IV, 3). Age and sex certainly affect levels of hemoglobin and of alkaline phosphatase, and probably affect others.

The following tentative summary of the use of analyses in cross-sectional studies of nutriture in individuals may be given. Estimations of ascorbic acid and of carotenoids in serum behave similarly in that both reflect the immediate past intake; a zero value of either is compatible with full health (in the case of carotenoids, a zero value of those that are biologically active corresponds to about 12 γ as usually estimated per

100 ml. of serum); and the information provided by the estimations may be very helpful for dietary purposes but is not of much value for assessing nutriture. Serum vitamin A falls early in deficiency and therefore estimations are valuable although individual variations are large. Blood pyruvate, blood hemoglobin, and serum phosphatase (in children) alter fairly early in deficiency; more work is required upon serum alkaline phosphatase in children and in adults before much can be said about this somewhat mysterious "enzyme." There is probably a slight drop in total protein and in albumin fairly early when the diet is low in protein, but in cross-sectional studies this does not help to assess the nutriture of an individual person; and some undernourished persons have unusually high values. A fall in serum calcium is a late occurrence. In living tissues such as leucocytes the amount of ascorbic acid reaches low values only shortly before malnutriture occurs, and the same is probably true for thiamine, niacin, and riboflavin. Blood values for these three members of the vitamin B complex become low only very late in malnutrition and the same seems usually to be true of serum values for protein or albumin. It must be emphasized once more that, even when the obvious factors such as changes in blood volume are controlled, we remain profoundly ignorant of the nondietary causes of variations between individuals at the same time or in single individuals at different times.

4. Composition of Urine

Though we are ignorant of the interpretation of blood values in man, we are even more ignorant of the interpretation of urinary values. Estimations are done sometimes upon random samples of urine, sometimes on fasting specimens, sometimes on 24-hour specimens, and sometimes after one or more test doses. The methods used have been briefly summarized elsewhere (Sinclair, 1947a). The urinary excretion of water-soluble vitamins will in general reflect the immediate past intake, but this has no clear relation to the assessment of nutriture. There seems to be no reason why a person in full health should be expected to excrete any ascorbic acid: he is not expected to excrete glucose in the urine. We know little about the factors involved in load tests (in which the excretion after a test dose is followed) or saturation tests (in which the number of days needed to "saturate" the body is determined); until we know more about the metabolism of nutrients and the renal threshold for them, the results of load or saturation tests must be interpreted with caution; a novice indulging in a glucose saturation or load test might reach very remarkable conclusions. Johnson, who has had a large experience of urinary excretion tests, prefers estimations upon fasting urine to load tests in field surveys (Johnson *et al.*, 1945). Miller and Beach (1945)

concluded that the fasting excretion of ascorbic acid "fails as a measure of vitamin C status of human subjects"; for thiamine they preferred load tests to fasting estimations because "apparently there are complex factors influencing the fasting excretion which tend to vitiate the fasting excretion value as a reliable measure of the state of thiamine saturation of the subject"; they reached similar conclusions regarding riboflavin. Mickelsen *et al.* (1947) could not distinguish between intakes of 1 mg. or 2 mg. of thiamine by urinary analysis. On the other hand, Cayer *et al.* (1945) concluded that urinary excretion tests for B complex vitamins "assist materially in evaluating the nutritional status of any given individual"; and Kajdi *et al.* (1939) obtained good results with a load test for ascorbic acid. The fact that only part of ingested niacinamide is excreted as N^1-methylniacinamide is a serious disadvantage in the estimation of this substance as a measure of the adequacy of niacin nutriture.

It has been customary, for reasons of convenience, to administer a single test dose in a load test, usually by mouth. Because many subjects in England during the war excreted no extra ascorbic acid after a test-dose of the usual size, we used large amounts administered orally (33 mg./kg. body-weight). Lowry and his colleagues (Lowry *et al.*, 1946), in an excellent study relating the concentration of ascorbic acid in different parts of blood and in urine to the dietary level, correctly point out that it is desirable to administer large amounts orally, but given in many divided doses throughout the day; this technique is often not practicable.

IV. Functional Assessment

1. *Introduction*

Physiology studies the functions of the body, and anatomy its structure. In this section we are not concerned with alterations in structure unless they affect function, but the two cannot be completely separated. Furthermore, the section on Clinical Assessment obviously must include consideration of function of which the testing (as in eliciting a knee jerk or counting the pulse) is a very common part of a clinician's examination. Arbitrarily we shall consider capillary fragility in this section, and radiological investigations in the section on Clinical Assessment.

2. *Visual Acuity*

It has been abundantly proved that rapid diminution can occur as a result of malnutrition. From the extensive Japanese literature, there is no doubt that retrobulbar neuritis occurs in association with beriberi (Kagawa, 1938), but the relationship to deficiency of thiamine is obscure. Diminished visual acuity has been alleged to be caused by deficiency of

riboflavin (Kruse *et al.*, 1940), and has often been described in pellagra together with retrobulbar neuritis and optic atrophy with central scotomata (e.g., Calhoun, 1918) although there is no clear evidence that deficiency of niacin causes the changes. Diminished vision was noted in warm climates by Strachan (1888), Scott (1918) and many subsequent authors as a symptom accompanying nutritional neuropathy. Such loss of vision caused by retrobulbar neuritis was described in Sierra Leone by Wright (1928), in Nigeria in 1930 by Moore (1939, 1940), in British Guiana in 1932 by Browne (1939), in Ceylon in 1933 by Nicholls (1935), in Malaya in 1935 by Landor and Pallister (1935; Pallister, 1940), in Trinidad by Métivier (1941). Grande and Peraita (1941) recorded visual disturbances in nearly half of a series of cases of nutritional neuropathy in Madrid; dimness of vision with retrobulbar neuritis and central or paracentral scotomata were found. Wilkinson (1944; Wilkinson and King, 1944) reported from Hong Kong cases of nutritional amblyopia which was painless and without scotomata. Spillane (1945, 1947) investigated nutritional retrobulbar neuritis in German prisoners in the Middle East; the discs were normal, slightly pale temporally or slightly hyperaemic, and there were central scotomata. Other cases were studied in Allied prisoners in the Far East (e.g., Adolph *et al.*, 1944; Rich, 1946).

The factor or factors concerned in nutritional visual disturbances in man are not known; deficiency of some part of the vitamin B complex (other than niacin, riboflavin, and possibly thiamine) is the most probable cause. It is well known that pigeons with acute deficiency of thiamine are blind, and that, since this sign is cured in a matter of minutes by injection of thiamine, it presumably arises from a biochemical lesion in the brain with no structural lesion. The visual disturbances in man, however, differ in that they are not immediately cured by therapy, and their etiology is almost certainly different.

The testing of visual acuity is important when there is a recent history of *rapidly* failing vision, such as marked impairment occurring during a period of up to about two months; otherwise its only use for our present purposes is as an aid in the interpretation of certain tests of rod threshold of the dark-adapted eye.

3. *Rod Threshold and Scotometry of the Dark-Adapted Eye*

Various workers have shown that when human volunteers partake of a diet very low in carotenoids and in vitamin A, but apparently adequate in other nutrients and in aliments, a significant increase in the rod threshold of the dark-adapted eye occurs after a variable period. The period, it is claimed, may be one of days (Wald *et al.*, 1938; Wald and Steven, 1939; Drigalski, 1939; Hecht and Mandelbaum, 1940), of weeks (Booher

et al., 1939; Pett, 1939), or of many months (Wagner, 1940; Wald *et al.*, 1942; Brenner and Roberts, 1943; see also Steffens *et al.*, 1940, and Isaacs *et al.*, 1940). The differences in the time that elapses before the functional abnormality appears cannot at present be fully explained. In the spring of 1942 Steven (1942) and other members of the staff of the Oxford Nutrition Survey conducted an experiment in which nine students at Magdalen College and I partook of such a diet for periods varying from 38 to 150 days. Full curves of dark adaptation after preliminary exposure to bright light, and the threshold of the dark-adapted eye, were determined at intervals using a slightly modified Wald machine (Wald, 1941) which itself is a modification of Hecht's machine. That machine has the advantage that its readings are relatively independent of visual acuity and of intelligence, and the subject cannot cheat the machine. Therefore closely reproducible results are obtained in a normal subject provided there are no marked variations in pupil diameter. Of the ten subjects, seven showed a significant deterioration in rod threshold of the dark-adapted eye, and at the same time there had been a significant fall in the amount of vitamin A in serum; two subjects approached the level of clinical night-blindness, one after 54 days and the other after 87, the rises being 0.75 and 0.8 log units respectively. Three subjects were treated with massive doses of vitamin A (halibut liver oil being used), and the rod thresholds and serum vitamin A were found to be normal a few hours later. It is concluded that the impaired rod threshold was almost certainly caused by deficiency of vitamin A, although it is just possible that some other factor in the halibut-liver oil may have produced the response.

Subsequently the Medical Research Council planned a similar experiment on much more extensive lines (1945). The Oxford Nutrition Survey, amongst others, again measured the serum vitamin A and determined the rod threshold of the dark-adapted eye using the same machine. This time deficiency as judged by the two sets of values occurred in none of the 16 subjects within a year, and only in 3 of 11 subjects after 14 to 22 months.

Similar unexplained variations in time of depletion and in response to therapy had been encountered by Wald and by Hecht. Factors as yet unknown are undoubtedly concerned in the utilization of vitamin A and carotenoids in the body, and in the rate at which the body becomes depleted; this may be fast, as in hyperthermia, or slow. What is certain is that deficiency of vitamin A can cause an impaired rod threshold in the dark-adapted eye, and that under certain conditions this will revert to normal a few hours after administration of fish liver oil rich in vitamin A. We urgently need further information about the metabolism of

vitamin A and of carotenoids in the body. Recent work in the Netherlands and Germany has shown that in malnutrition there may be impaired utilization of carotenoids apparently caused by a dietary deficiency.

Deficiency of vitamin A is, of course, not the only cause of an impaired final rod threshold of the dark-adapted eye. If the threshold is being used in the assessment of human nutriture, the first essential is to employ a reliable instrument calibrated at frequent intervals. If an impaired threshold is found, the eye should be examined for other possible causes of this abnormality such as retinal pigment, or opacities of lens or cornea. If no such cause is discovered, therapy with vitamin A should be given and the rod threshold then retested. If a significant improvement occurs, it is very probable that the subject was deficient in vitamin A. The Oxford Nutrition Survey has used the following method for the routine testing of large numbers of persons. Persons enter the darkroom through a door with a light trap at intervals of about 3 minutes. They sit in absolute darkness for at least 20 minutes and then readings of the rod threshold are made with the modified Wald machine and continued until consistent results are obtained. If, after slit-lamp microscopical and ophthalmoscopical examinations of the eye have been made, there is no explanation of an impaired rod threshold, the subject is given 20,000 I.U. of vitamin A orally on each of 7 days and then retested. If a significant improvement is then found, the subject is believed to have been deficient in the vitamin, but if the threshold is stable to therapy, deficiency is not excluded: there are reasons as yet unknown why subjects deficient in vitamin A do not rapidly respond to such therapy, although there are of course factors other than deficiency of vitamin A that will cause an impaired threshold in absence of observed structural abnormalities of the eye.

In this work an area 2.5° in diameter located 6° above the fovea has been used. A similar area 20° below the fovea has also been tested in a large number of persons and there is a very high correlation between the two sets of readings. Livingston, however, has shown (1944) that scotometry of the dark-adapted eye would reveal a diminished field of rod function in subjects on a diet deficient in vitamin A and carotenoids before our testing at 6° above the fovea showed impaired function. It is probable therefore that scotometry detects deficiency at an earlier stage, but it is not practicable in routine assessments of nutriture.

4. *Capillary Fragility*

Measurements of capillary fragility have been made by modifications of two methods, that of Hecht (1907) in which a reduced pressure was applied to the outside of the capillaries, and that of Göthlin (1931) in

which an increased positive pressure was produced within them. The former method is the better and can conveniently be used on the capillaries of the skin by applying a cup, 1 sq. in. in area, to the front of the forearm just below the antecubital fossa and suddenly reducing the pressure by 200 mm. of mercury for 1 minute; the difference in the number of petechiae in the area before and after the reduction in pressure is estimated by viewing the area through a lens of about ×15 diameters. Using this method we have found no correlation between capillary fragility and the amount of ascorbic acid in serum or in blood (thereby confirming earlier work of Deggeller, 1936; Liebmann *et al.*, 1938; Rapaport *et al.*, 1940; Scarborough and Gilchrist, 1944; and others) or in leucocytes or erythrocytes, or the fasting excretion of ascorbic acid, or the amount of a test-dose excreted. This merely supports the now general view that the earlier claims for the use of this test in the diagnosis of deficiency of ascorbic acid are erroneous. One striking demonstration of this error was that when Crandon had induced "frank scurvy" in himself by partaking of a diet deficient in ascorbic acid, both the increased and reduced-pressure tests gave negative results (Crandon *et al.*, 1940). In the last few years the work of Scarborough (1945), Bacharach *et al.* (1942) and others has indicated that in lower animals and possibly in man the test is useful in estimating deficiency of vitamin P.

The fragility of capillaries in the gums was possibly a more hopeful measurement, conveniently made by applying a reduced pressure to the gums at the base of the interdental papillae through an auriscope. Our results with this however have not been encouraging as an indication of deficiency of ascorbic acid.

5. Nerve Accommodation and Chronaxie

Various French authors have included measurement of chronaxie in an attempt to detect early neuromuscular disorders of nutritional origin. In work that was not carefully controlled, Elsom *et al.* (1940) claimed that 3 days after she started a diet low in the vitamin B complex, there was "diminution in electrical irritability of the peripheral nerves." According to Lecoq *et al.* (1946), "Dans l'appréciation des troubles neuromusculaires latents des avitaminoses, la méthode chronaximétrique constitue un test précieux qui permet de mettre en évidence la carence avant toutes manifestations cliniques." In lower animals deficiency of vitamin E increases chronaxie (Victor, 1934), but there is no evidence that this happens in man.

The measurement of nerve accommodation, rather than of chronaxie, would presumably find greater favor outside France, but insufficient work has been published to permit of an assessment of its use. In the

joint experiment already mentioned in which human volunteers were placed upon a diet deficient in thiamine for 86 days, some receiving supplements of thiamine daily, measurement of nerve accommodation at a time when a clinical examination was useless for distinguishing the two groups could produce a successful differentiation between deficient and control subjects in about 70% of them; estimations of thiamine in blood could at this time, as previously mentioned, differentiate the two groups with complete success. It is probable that the measurement of nerve accommodation in deficiency of thiamine will prove to be of value similar to that of the measurement of rod threshold in deficiency of vitamin A: impairment of function will be detected earlier than by the usual clinical examination, but nutritional deficiency will be only one possible cause of such impairment.

6. Ergometry, Endurance, and Ischemic Pain

Tests of physical fitness, of capacity for physical work or of fatigue have been used in the assessment of nutriture; they have been studied particularly in subjects placed experimentally upon deficient diets. In such work reliance can seldom be placed upon the reports of the subjects themselves or the subjective impressions of observers. Therefore carefully controlled tests have been introduced, particularly by Keys in Minneapolis and Johnson in Boston.

The performance of work by muscles is dependent in part upon the supply of aliments for yielding energy by metabolic changes, the supply of nutrients that take part in such changes, and the maintenance of homeostasis by counteracting chemical and physical processes resulting from such changes. Care, however, must be used in interpreting results: if administration of disodium hydrogen phosphate is found to diminish muscular fatigue or increase performance of work, it would not necessarily be correct to assume that the person was previously deficient in phosphate. Further, the performance of muscular work and the onset of subjective fatigue are greatly affected by motivation and by training. There is no doubt that both of those are affected in severe undernourishment, in anemia, in beriberi, in pellagra, and in scurvy; the problem is to find whether malnutriture is limiting performance of muscular work or increasing fatigue in an individual or group of persons. No single test is likely to achieve this since individual variation is so great, and therefore recourse must be made to comparison of groups, or to supplementation of the diet or, if the particular problem permits, to tests made on subjects placed upon deficient diets.

Since "endurance is apt to be a quick and sensitive indicator of nutritional inadequacy" (Keys, 1946), particularly of undernourishment,

tests of endurance are likely to be useful in nutritional surveys. But in these it is not easy to employ the type that is used in experimental laboratory research. Milligan (cf. Milligan and Lewis-Faning, 1942) introduced the bar test for children: the child hangs by the arms for as long as he will from a horizontal bar; the time is recorded and this or the product of multiplying it by body-weight is used as a measure of nutriture. Unfortunately motivation is a very important factor in a test such as this with children: if two or more children hang from the bar simultaneously, they will tend to be much "better nourished" than if they hang singly. When we applied this test to undernourished boys in Holland, we obtained shorter times than Milligan got with English boys; but it is doubtful to what extent the values under the different conditions obtaining in the two countries can be compared. Any test of endurance should test strength and motivation to as slight degrees as possible.

Though certain claims to the contrary have been made on somewhat inadequate grounds, the evidence is that no gain in fitness of adults occurs when vitamin supplements are added to a good American diet (Keys and Henschel, 1942; Foltz *et al.*, 1942; Ruffin and Cayer, 1944; Archdeacon and Murlin, 1944; Borsook *et al.*, 1946). Similar results were obtained in Britain during the war (Bransby *et al.*, 1944), but Kruse *et al.* (1944) have pointed out that under the conditions used the length of the study, which would correspond to 10 or 11 days in the life of a rat, might not be expected to permit of demonstrable improvement.

It might be supposed that vitamins of the B complex would play the most prominent part amongst the nutrients in affecting fitness or fatigue, since certain of them have been proved to take part in metabolic changes that yield energy. The Boston workers have claimed (Egaña *et al.*, 1942; Johnson *et al.*, 1942) that a diet deficient in vitamins of the B complex produces physical deterioration in sedentary men within four weeks and marked reduction in capacity for work in men doing hard work within a few days; but Keys (1943a) has pointed out that "after careful consideration these claims must be rejected in toto." In controlled work Keys and his colleagues found "no effect whatsoever of a very severe restriction in B vitamins coupled with hard work when this is carried on for a matter of a couple of weeks" (Keys, 1943b); the first notable effects were anorexia and mental depression, and some of the subsequent loss of fitness may have been due to undernourishment and tended to be corrected by thiamine alone (Keys *et al.*, 1945). On diets supplying 34 to 40% of the N.R.C.'s allowances of the major B vitamins, there was no observed loss in physical or mental capacity within at least 6 months (Keys *et al.*, 1945; Cogswell *et al.*, 1946). However, Barborka *et al.*

(1943), in well controlled work on four subjects, claimed that a diet low in thiamine (0.65 mg. daily) and riboflavin (0.94 mg. daily) "produced a definite decrease in work output shortly after it was begun"; and Archdeacon and Murlin (1944) obtained on a diet low in B complex vitamins greatly decreased muscular endurance that was markedly improved by thiamine but not by riboflavin. There are many claims (Csik and Bencsik, 1927; Morell, 1940; Gounelle, 1940; McCormick, 1940; Droese, 1941; Rosenbaum *et al.*, 1942) that the performance of work by individuals who show no signs of deficiency is increased by administration of vitamins of the B complex, but these have not been properly substantiated. A diet low in riboflavin was found by Keys *et al.* (1944) to produce no change in fitness in 6 months.

Since the onset of pain in a group of muscles exercised under controlled conditions is highly reproducible (Lewis *et al.*, 1929–31), and since this pain is presumably caused by the accumulation of some metabolite, we have tested the effect upon the onset of ischemic pain of injections of thiamine in normal subjects, and of experimentally induced deficiency of thiamine. Neither excess of thiamine nor deficiency influenced the onset of ischemic pain under the conditions of our tests.

7. *Dynamometry*

The force exerted under given conditions by groups of muscles has been measured by certain workers in the belief that this measurement would assist in the assessment of nutriture. A dynamometer that measures lumbar pull has been most frequently used. There is, however, enormous variation in strength between individuals; therefore cross-sectional studies are not likely to prove of value. And longitudinal studies when subjects have been placed upon deficient diets have shown that strength is very resistant to brief periods of deprivation: Brozek *et al.* (1946) found only a slight deterioration in strength in acute deprivation of vitamins of the B complex; and Keys and his colleagues also found that strength was much less affected than was endurance by undernourishment, although obviously strength is reduced when there is atrophy of muscles.

In nutritional surveys it would seem that no useful purpose is served by measurements of strength.

8. *Cardiovascular and Respiratory Function*

In undernourishment there is sinus bradycardia which may produce pulse rates below 40 beats a minute, or even below 30. There is also a fall in both systolic and diastolic blood-pressures, and an increase in circulation time. In deficiency of thiamine, on the other hand, there is

usually tachycardia, low diastolic pressure, and a decrease in circulation time with peripheral vasodilatation.

In the assessment of nutriture measurements of pulse rate and blood pressure are of value; measurements of vital capacity or of breath holding are worthless. Elaborate tests of cardiovascular or respiratory function, such as the pulse rate after standard exercise, are not usually practical.

9. *Psychometric Tests and Coordination*

As a result of his studies on acute deficiency of vitamins of the B complex, Keys (1947) concludes that intellective capacity and the special senses are remarkably resistant, and psychomotor functions like neuromuscular coordination and speed are less affected than endurance. Coordination and steadiness greatly deteriorate in brief deficiency of aliments (Taylor *et al.*, 1945) or in prolonged semistarvation; whereas fasting for a few days produces a far greater decline in coordination than in strength, prolonged semistarvation produces greater losses in strength than in coordination (Keys, 1946).

Intellective functions are relatively resistant to nutritional deficiencies, although mental derangement occurs late in pellagra. As Keys (1946) points out, the claims of Harrell (1943, 1946) regarding the effect of added thiamine on learning in children are unacceptable.

V. Somatometric Assessment

It is obvious that if there is an inadequate supply of the substances needed for the formation of new tissue during body growth, this will be limited; limitation may also occur if nutriture is disturbed by deficiency of other aliments or nutrients. This simple fact has been appreciated for a long time, and therefore much attention has been paid by nutritionists to the growth of children. But insufficient thought has been given to the most desirable rate of growth, which is not necessarily the maximum rate. We can make a boy of 12 years taller and heavier than he would otherwise be by injecting anterior pituitary lobe extract; alternatively we can make him heavier and probably taller by superalimentation. There is indeed a tendency amongst nutritionists to regard the child of perfect nutriture as placid, rotund, red faced, and seated in contented contemplation of its folds of flesh. During the past several years there has been a marked increase in the rate of growth of children, although in England and the U.S. the adult male height has remained unchanged. It has not been shown that this increase in rate is necessarily advantageous; indeed it may be undesirable since the long time taken to reach maturity is characteristic of the human genus. It is known from the work of Wolbach that in young rats and guinea-pigs fed on diets containing large

amounts of vitamin A there is a great acceleration of bone growth so that "it is possible to get the equivalent of a year's growth in a ten to fifteen day period" (Wolbach, 1945); the new bone is inadequately calcified and fractures easily. But a great acceleration of growth of this type seems to be the goal of many nutritionists despite its inconveniences.

The factors that condition growth include the following: heredity (including race and sex) and somatotype; birth conditions; age; geographical and temporal circumstances; activity; illness; obesity; nutrition. The use of some body measurement, or measurements, dependent on growth for the cross-sectional assessment of nutriture in an individual is therefore complex. In fact the unknowns are so many that the attempt is useless. A child with scurvy may have normal body measurements; rickets is a disease of growth, and athreptic infants that are not growing tend not to develop rickets; persons with famine edema may be statistically normal in weight even when allowance is made for the extra fluid. Recognition of the uselessness of sex-age-height-weight tables led to various other body measurements and a variety of indices being introduced. For instance, W/H^3 was used by Buffon in 1829, by Quetelet in 1836 and by Röhrer in 1908; other W/H indices were used by Tuxford (1917). Stem length was related to weight in von Pirquet's "Pelidisi" index, and to erect height in Manouvrier's index. Such cross-sectional data on single individuals are worthless.

After trying unsuccessfully to assess nutriture by weight, Oeder (1910) used the thickness of skin and subcutaneous tissue over the abdomen. Kornfeld and Schüller (1930) chose four sites (chest, abdomen, back and cheek). Mitchell (1932) used the excellent regression equations derived by Franzen (1929). Believing that the thickness over the arm is less affected by somatotype than that over the abdomen, we take the thickness at constant pressure of a fold of skin and subcutaneous tissue measured longitudinally over the top of biceps and midway down the back of the forearm; apart from depending on somatotype, the measurement is affected by age and sex, but it gives an indication of "thinness" which may be relevant mainly to aliment nutriture. It is obvious, however, that the athlete will tend to be "thinner" by this criterion when in training than when he is engaged in sedentary pursuits.

Somatometric methods can be very valuable, indeed, essential, to certain longitudinal studies, but these are not the main concern of this article. In our surveys in Germany we find it convenient and profitable to follow monthly the changes in weight of individuals and also of random samples of the population. For adults, the results are most easily computed and compared with other groups if the weights are first corrected for height. (It would be more satisfactory in theory to correct

weight for stem length.) But even in longitudinal studies of the body weight of individual adults, severe extraneous factors interfere. Such are the customary seasonal variations in nude weight, the greater seasonal variations in clothing if worn for weighing, and variations in the fluid content of the body (as in famine edema). In children there is the additional complication of growth spurts. It is well known that there are two accelerations in growth: the mid-growth spurt occurs from about the ages of $5\frac{1}{2}$ to $7\frac{1}{2}$ years in both sexes and takes place mainly by accelerated increases in girths; the adolescent spurt occurs in well-nourished North American girls from about 9 to 13 years of age and in boys about 2 years later, and is mainly accelerated increases in stem length. Unfortunately there are variations in the age of onset and in the duration of the spurts, which complicate a simple grid method of comparing actual growth with "normal" growth as is attempted by Wetzel (1941, 1943). Growth of nails (le Gros Clark and Buxton, 1938) and growth of hair are undoubtedly affected by nutrition, but have not been studied adequately.

VI. Clinical Assessment

1. *Mortality and Vital Statistics*

A physical examination is essential in the proper assessment of nutriture. But unfortunately clinical findings are harder to interpret and have been subjected to more fallacious interpretation, than any other group of data that we are discussing.

In comparing the nutriture of different populations, statistics of mortality have been used. As Jolliffe and Most (1943) point out, the usual mortality statistics give a very inadequate indication of the incidence of severe nutritional diseases. Deaths from certain diseases that are influenced by nutrition, such as pulmonary tuberculosis, or morbidity statistics concerning the incidence of such diseases are of some interest but are usually very difficult to interpret because of variations in other relevant factors. For instance, an apparent increase in the incidence of pulmonary tuberculosis (judged by the present returns of "active" cases or of deaths from the disease) in the British Zone of Germany as compared with 1939 might be due to a variety of causes including different criteria used in the returns, overcrowding, inadequate heating, or malnutrition.

The difficulty of interpreting certain statistics is also shown by studies of the effect of nutrition on complications of human pregnancy, childbirth, and infancy. This effect has been studied experimentally by three groups of workers: the National Birthday Trust Fund (Williams, 1937a, b, 1938, 1939; Balfour, 1938, 1944); the Toronto group (Ebbs *et al.*,

1941, 1942a, b); and the People's League of Health (1942). Although there are statistical objections to some of this work, the general results are impressive; a review of this work cannot be undertaken here. Baird (1945) has pointed out the importance of nutrition in prematurity and stillbirths, and Sutherland (1946) in the latter. Smith (1947a, b), however, could find no effect of the Dutch famine upon stillbirths or neonatal mortality, and no increase in toxemia.

2. *Methods of Examination*

An important feature of the physical examination is the differentiation of structural abnormalities that can be inspected. For this purpose various aids have been introduced. A loupe is of course customarily used by dermatologists and ophthalmologists. A very useful instrument for examining the skin and tongue is the type of illuminated magnifier used by thread provers and having a magnification of 10 or 20 diameters. For instance, the papillae of the tongue of a group of persons may be rapidly inspected with such an instrument by inserting microscope slides between the instrument and the tongue.

For higher magnification, the biomicroscope is used. We have modified an ordinary type of slit-lamp microscope so that it can be tilted downwards to view from above structures such as the tongue. The skin over the triceps area is conveniently inspected with any instrument if the patient stands with his back to the observer and rests his elbow in the chin-rest. For inspection of the limbus of the eye, the slit should be opened slightly, and the vessels examined by reflected light. This is greatly helped by the use of polarized light, produced by placing a disc of polaroid over the lens that focuses the slit. Plane polarized light makes the definition of the scleral-conjunctival margin very sharp, and also assists the appreciation of conjunctival opacity; the use of a second polarizer on the objective of the microscope enhances the effect but makes the retroillumination of the vessels too dim.

The interpretation of abnormalities of the limbus, together with that of other clinical signs, will be discussed below. Because of the importance of therapeutic trials in the interpretation of such signs, a note concerning them may be inserted here from our instructions for the use of teams in Germany.

Therapeutic Trials. It is obvious that the mere listing of the incidence of certain clinical signs (such as magenta tongues or folliculosis or gingivitis or pallor) is not of practical use unless some estimate of the etiology can be made. It is not only unscientific but actually misleading to attribute such signs to deficiency of particular nutrients unless evidence is available. Biochemical estimations, and even dietary investigations,

help in this, but when practicable therapeutic trials should be instituted. A group of subjects showing the lesion is selected and carefully examined, if possible an objective record of the lesion being obtained. The subjects are divided into two subgroups, one being given the therapeutic substance and the other no therapy (or preferably a placebo). The subjects are examined again after a suitable interval and at this examination it is essential that the clinician should not know which of the subjects are test and which controls. After the findings have been recorded on each subject, the results can be assessed. It should of course be pointed out to the subjects that something wrong with their health may have been found and that the treatment is being instituted to show whether this is so; at the re-examination it must be clearly established that the therapy has been followed, and the best indication of this is often afforded by a repeat biochemical examination.

When such therapeutic trials are carried out, and on other occasions, it is useful to make an objective record of the lesion. Several groups of workers have used skinprints or tongueprints (Sinclair, 1945). Excellent cameras have become available for making records, but a disastrous tendency has arisen to use them as a substitute for, rather than as an aid to, the clinician's examination. The RCAF ophthalmic camera (Tisdall *et al.*, 1943) is perhaps the best of such machines, but I have seen a print from it misinterpreted: in a survey in Northern Canada I had examined biomicroscopically the limbuses of the eyes of a Canadian Indian and pronounced them normal; but the dots of pigment that outlined the scleral digitations in this dark-skinned individual were interpreted in a photograph as being erythrocytes in abnormal limbal vessels and the individual was therefore pronounced by another observer to be deficient in riboflavin.

Whenever possible, of course, actual measurements should be made in the course of the examination, rather than qualitative observations. This is particularly important when different types of population are being examined because of the extreme difficulty of maintaining the same criteria: if a sign is found to be common in a particular population, but was rare in one previously examined, there is a tendency to alter one's criteria and grade grosser degrees of the sign.

Many signs can be quantitatively recorded if this is thought desirable. For instance, gingival tenderness can be measured by pressure with a blunt probe. It is easy to construct a spring-loaded probe that will give measurable pressure. The normal gum papillae should withstand a pressure of 1 kg. on 7 sq. mm. (14 atmospheres) without pain or bleeding. Such measurements are often made by physiologists in the study of the normal but rarely by clinicians in their study of the abnormal.

For instance, Ryle (1947), in Oxford, has classed the strength of the knee jerk and the heart sounds as "immeasurables"; the former was measured by Franklin (1938) in Oxford, and the latter are frequently recorded by physiologists using a stethocardiograph.

It is possible to examine briefly in nutritional surveys for public health purposes large numbers of subjects in a short time if a routine is adopted. Our usual method is as follows: Males and children up to the age of 12 are examined unclothed to the waist and without shoes and socks; females (except girls under 12) are examined without shoes and stockings and with the whole of one arm bare. While the subject is sitting, the lips, teeth and gums, and tongue are examined; this is followed by the pulse, skin of the arms, muscle irritability, thickness of skin and subcutaneous tissue, vibration sense, edema, skin of the legs, calf tenderness, and jerks. The subject then stands and the following examinations are made: heart, lungs, and skin of the back of the chest and triceps (abdomen for enlarged liver or spleen if these are to be felt in children in the standing position), eyes and skin of face, posture, thinness. Then follow any special biomicroscopical examinations. Finally, any comments are made about the mentality of the subject and his nutriture, and then the diagnosis is recorded.

3. General Assessement by Inspection

At the beginning of this century attention began to be paid in various countries to the assessment of nutriture by a rapid inspection of (usually) children. In England, for instance, the medical inspection of schoolchildren was introduced by the Board of Education in 1907, and in this the assessment of nutriture played a part. Such examinations usually assessed physical state, of which health is one component; and nutriture is a component of health: it is not surprising therefore that they were often only remotely connected with nutriture. When nutriture was separated from the other components, it was often identified with growth in relation to age, or with amount of subcutaneous fat (Koppe, 1905) with or without extent of muscular development (Hogarth, 1909), or pallor (Gastpar, 1908). When nutriture was not so distinguished, it often became synonymous with physical state, as in the Dunfermline system (Mackenzie, 1913). In this, which formed the pattern of much subsequent work, the following were taken into account: height and weight in relation to age; general appearance; mucous membranes, skin and subcutaneous tissue; muscular tone and development; facial expression, posture, movements, voice, interest and attention.

After the First World War, von Pirquet with Nobel and others intro-

duced the Sacratama index which rated the following: the blood content of the skin, the amount of subcutaneous fat, the skin tension determined by the water content of the subcutaneous tissue, and the condition of the muscles. The Berlin conference of the League of Nations recommended (1933) that a "general impression" of the condition and constitution of persons examined should be recorded, and for this purpose they recommended von Pirquet's methods.

In 1934 the Board of Education in England issued instructions (Admin. Mem. No. 124) for the use of School Medical Officers in their assessment of nutriture which was not to be based solely on height and weight. Children were put into four classes (excellent, normal, slightly subnormal, bad) in an attempt to determine undernourishment on the basis of: general appearance; expression; posture; tone and function of the muscular system; condition of the skin and hair; color of the mucous membranes; amount of subcutaneous fat; teeth; presence of any defect or deformity. In 1937 the Advisory Committee on Nutrition of the Ministry of Health concluded that they could not recommend as reliable any of the known methods of assessing nutriture, but they considered the above method of the Board of Education to be the most promising; the Committee believed "that research should be continued to establish, if possible, a reliable test or group of tests for the assessment of the state of nutrition." There are three pertinent questions regarding the Board's method: first, whether it in fact assesses general nutriture rather than muscular development and physique; secondly, whether experienced School Medical Officers are consistent one with another in their grading of children; thirdly, whether one such experienced doctor is consistent with himself on different occasions. The last two questions were excellently investigated by Jones (1938) who obtained very definitely negative answers to them. For instance, in one experiment in which five doctors examined the same 193 boys and re-examined them a week later, "on both occasions in more than half the cases, the doctors failed to agree over the crucial question of whether a boy is satisfactorily nourished or not," and one doctor found 1.5% to be subnormal or bad whereas another doctor put 47% in these classes. And the doctors' results at the second examination were not consistent with their results at the first. Jones's correlations between the ratings given by the doctors and various somatometric indices make it extremely probable that the doctors were strongly influenced by the height and weight of the children. Jones found that "On the average, the experienced School Medical Officer picks out two-thirds, and [Tuxford's height-weight] index three-quarters, of the boys assessed subnormal by two or more doctors." It seems that the Board of Education assessment has no advantages, and certain disad-

vantages, over simple records of height and weight, and these cannot alone be satisfactorily used to assess general nutriture.

Recently some results of surveys made by the Ministry of Health clinical team have been published (Adcock *et al.*, 1947); in this work five clinicians have at different times taken part. This paper contains certain errors, and I have mentioned elsewhere some that occur in it (Sinclair, 1947d). The authors have used two types of clinical methods. First, the clinician placed the subject into one of three grades—"good," "fair," "poor"—according to his "general state of nutrition." Secondly, the presence of 29 "clinical signs" was recorded. In this paper the findings on 3351 children aged 8–15 years and on 3326 adults are analyzed statistically to ascertain whether any relationship exists between the incidence of the "clinical signs" singly or collectively, and "the clinician's nutritional grade." The incidence of each clinical sign is also compared with "the nutritional state as a combination of all the clinical signs." The authors conclude that "nutritional assessment as at present understood is determined mainly by the value placed on the grading criteria . . . and to a negligible extent or not at all by the presence or absence of clinical signs." The "clinical signs" (which are sometimes also called "nutritional signs") include "dandruff," "pityriasis," "acne," "ichthyosis," "pinguecula," "oedema"; it is obvious therefore that they include many signs that are not nutritional in origin, and the term "nutritional signs" should not be used for them, particularly as the authors conclude that the signs play little if any part in the "nutritional assessment as at present understood." The authors use for "the nutritional state as a combination of all the clinical signs" the term "the general nutritional factor"; from this it follows that an adolescent with a marked seborrheic diathesis will have a very poor "nutritional state," scoring under "seborrhea," "dandruff," "acne," and perhaps "pityriasis"; whereas an adolescent with gross undernourishment (thin, pale, wasted, with inelastic skin) will probably not have any "nutritional sign" recorded and therefore will be in an excellent "nutritional state." The authors state that the clinical signs were "not taken into account in grading the subjects according to their state of nutrition." Yet one such sign, edema, occurs also in the list of grading criteria. It is possible for a child to have scurvy, and have none of the grading criteria; yet it would be hard indeed for the clinician to put such a child in the class of "good nutrition." Further, the paper to which the authors refer for further details (Magee, 1944) specifically attributes the higher incidence of children graded "poor" in these surveys, as compared with the incidence in the examinations of the School Medical Service, to the "more elaborate methods of diagnosis used by the Ministry's observers" including the slit-lamp

miscroscope; therefore the clinical signs found in the eye with this instrument (e.g., corneal vascularization) were in fact used in assessing "nutritional grade" in contradistinction to the statement in the present paper. It is rather remarkable that corneal vascularization is apparently commoner than dandruff or acne in adolescents, or than pingueculae or pyorrhea in adults—the more remarkable as two of the clinicians, Drs. Sydenstricker and Stannus, have in their publications wisely adopted a conservative attitude about what constitutes invasion of the cornea. Since the authors find no correlation between the signs singly and "an internal factor (the general nutritional factor) responsible for the signs," they conclude that the clinical signs play a negligible or no part in the assessment of nutriture. This conclusion is clearly not justified since there is obviously no single internal factor responsible for the signs; and even if all of them were caused by nutritional deficiencies, the varied combinations of signs in different deficiencies would preclude such positive correlations. The authors find a high degree of correlation of these selected "grading criteria" with "nutritional assessment"; for instance, a child that is abnormally thin is likely to be graded "fair" or "poor," and not "good." From this the authors conclude "that the grading criteria are the main elements in the diagnosis of nutritional grade" (which is so by definition). It is apparent that the authors consider that the clinician usually regards a child with poor posture as being in a poor nutritional state, whereas a child with scurvy may well be regarded as being in a good nutritional state. We see but once more that this type of so-called nutritional assessment, whereby persons are placed in categories on the basis of posture and expression and the like, is worthless as an assessment of general nutriture. And we also find in this study no reason for doubting the value of recording true clinical signs of malnutriture.

Unfortunately the quest for such true clinical signs is made difficult by their unspecificity. And therefore a large part of this article must be devoted to a discussion of them.

4. Symptoms

A medical history is essential in the proper assessment of nutriture. Complaints volunteered by the person should first be investigated and past history obtained with particular reference to illnesses in the past year. Then information regarding certain specific symptoms should be sought. The most useful are: change in body weight, muscle pain or cramps including pain on rising from a chair, sore tongue, sore eyes, rapidly failing vision, paresthesia, weakness or fatigue, nycturia, diarrhea; information about menstruation and lactation should be obtained when relevant. We have found a history of edema almost useless: when asked

the usual questions some persons will give a negative answer even when they have marked pitting edema, whereas others will with no justification complain for instance of tight shoes. In general, symptoms must be accepted with great caution. One person will suffer in stoic silence grave insults to the body; another will whine about trivial or even imaginary ills.

5. Signs

It is perhaps fortunate that epithelial tissues are affected more than are mesenchymal by nutritional deficiency, and of the epithelial tissues the epidermis, the hair and the cornea are affected more than are others. Since these three are easily inspected, the elucidation of clinical signs is assisted. It might of course be supposed that the ease of inspection is the cause of the more numerous descriptions of lesions in these tissues; but histological studies of organs in deficiencies in both man and other animals have made this unlikely.

Skin, Hair, and Nails. In the assessment of nutriture of groups of the population the following should be recorded regarding the skin and its appendages.

i. Extent of examination. (It is often forgotten that the larger the area of skin inspected, the greater the chance of finding abnormalities; further, the depigmentation of kwashiorkor occurs around the anus, folliculosis commonly upon the buttocks, and the dermatitis of ariboflavinosis around the external genital organs. Therefore if populations or individuals are to be compared, the extent of the examination must be the same.)

ii. Pallor, suborbital pigmentation, generalized pigmentation (and depigmentation), erythema, ecchymoses, perifollicular petechiae.

iii. Eczema, "sores" and unhealed lesions, nutritional seborrhea, pellagrous dermatosis, crackling, pityriasis sicca, generalized xeroderma, desquamation.

iv. Diminished elasticity, atrophy, thickening, folliculosis, follicular hyperkeratosis.

v. Folliculitis, infestations.

vi. Hypochromotrichia. Other hair abnormality.

vii. Onychodystrophy.

Deficiencies of at least 11 nutrients probably cause alterations in the epidermis in mammals; and because of the confusion regarding such lesions in man, those occurring in the early stages of deficiency in the rat will be briefly summarized following the excellent studies of Sullivan and others.

In the rat, deficiency of vitamin A (Sullivan and Evans, 1945) causes

keratinization of the epithelium and hair follicles with no changes in the corium and sebaceous glands. Deficiency of linoleic or arachidonic acid (Williamson, 1941) causes hyperkeratosis of the epithelium with necrosis of the tip of the tail and scaliness of the dorsum of the paws. Deficiency of riboflavin (Sullivan and Nicholls, 1941) causes atrophy of the skin and appendages with alopecia. Hyperkeratosis and acanthosis are caused by deficiencies of pyridoxine (Sullivan and Nicholls, 1940), pantothenic acid (Sullivan and Nicholls, 1942a) or biotin (Sullivan and Nicholls, 1942b); in the first, there is also marked hyperemia and edema ("rat acrodynia"), in the second there is also dilatation of the hair follicles accompanied by greying of the fur and some alopecia, and in deficiency of biotin there is also alopecia. Greying of the fur is also caused by deficiency of *p*-aminobenzoic acid (Ansbacher, 1941). Deficiency of magnesium causes hyperemia of the corium, particularly of the ears and paws with hyperkeratosis later (Sullivan and Evans, 1944), and deficiency of zinc causes hyperkeratosis with atrophy of the hair follicles and later hyperemia of the corium (Follis *et al.*, 1941). This, however, is in rats. In mice, deficiency of inositol causes alopecia (Woolley, 1941). These results on lower animals cannot necessarily be applied to man.

The commonest change in lower animals is hyperkeratosis, often with acanthosis and rarely with parakeratosis. In man there are two broad types of hyperkeratosis caused by nutritional deficiency: in one it is limited to the hair follicles, and in the other it occurs in areas subjected to trauma whether by irradiation or pressure. The former includes different types of lesion as previously pointed out (Sinclair, 1945). One type, folliculosis, consists of hyperkeratosis surrounding the mouth of the follicle and forming a plaque that covers the mouth and imprisons the hair which remains coiled beneath it or may burst forth carrying the plaque and superficially resembling a spine; it is found particularly over the buttocks, thighs, calves, and upper arms. Folliculosis can undoubtedly be caused by deficiency of ascorbic acid; many of the early accounts of scurvy mention it, and proof was afforded by Crandon (Crandon *et al.*, 1940). Follicular hyperkeratosis, in which a spine often projects from the follicle alongside the hair, is alleged by many authors to be caused by deficiency of vitamin A and by others to be pathognomonic of such deficiency. The first claim is probably true but lacks rigid proof; the second is false. Hyperkeratotic papules without a spine are common at puberty, particularly in obese people; in obese women they tend to persist and often are erythematous. An important point is that in these cases, in which there is no dietary deficiency, there is usually no generalized xerosis of the skin. This contrasts strongly with a very distinct form of follicular hyperkeratosis that is found in malnutrition and which

resembles goose-skin due to cold even in having erect hairs and is accompanied by generalized xerosis; it may perhaps be caused by deficiency of vitamin A or of unsaturated fatty acids (and these are almost always administered with so-called "specific" therapy with vitamin A since they are contained in fish-liver oils and arachis oil), and it can certainly be caused by deficiency of aliments.

The second form of hyperkeratosis, namely, the generalized type, can be caused by chronic deficiency of niacin. The thickened skin, which may not be pigmented, is found most frequently over bony prominences and also the palms of the hands and soles of the feet.

Erythema of the epidermis is another striking manifestation of nutritional deficiency in lower animals, and it occurs in certain deficiencies in man. Whether the primary lesion is in the cells of the epidermis or in the cells of the minute vessels supplying it is a question that need not now detain us, but passing reference will be made to it later. In acute deficiency of niacin erythema, which occurs in areas subjected to mild trauma, is the first visible change; there is dilatation of the vessels in the superficial corium and hyperkeratosis with parakeratosis in the overlying epithelium; the sebaceous glands atrophy. Other changes occur later, giving for instance the cracked pigmented type of skin found particularly on the shins. Two other changes that occur particularly over the shins are a fine branny desquamation and thin shiny atrophic skin; the former which we saw frequently in Germany after the war occurs not uncommonly in malnutriture and can also be caused in the exceptional circumstances of biotin deficiency (Sydenstricker *et al.*, 1942). Dry scaly erythematous patches, pityriasis sicca, occur on the face of children particularly in the areas where secretions such as tears and saliva tend to accumulate; they may be caused by nutritional deficiency or may be related to deficiency of soap. Neither acrodynia nor acne rosacea is caused by nutritional deficiency. Erythema is also part of the seborrheic dermatitis found in deficiency of riboflavin; this affects particular regions such as the hair margin on the head, the malar eminences, and the scrotum, and also mucocutaneous junctions. In the rat deficiency of riboflavin causes the sebaceous glands at first to hypertrophy and then to atrophy (but in the mouse there is no change in them); it seems that in man also there is an alteration in these glands. Hemorrhages in the epidermis occur in deficiencies of ascorbic acid and of vitamin K. In the former, characteristic perifollicular petechiae are produced; in the latter, ecchymoses. It seems to be not uncommon for bug-bites to be attributed to deficiency of ascorbic acid; and a lady with perifollicular erythema and folliculosis was once demonstrated to me by a distinguished professor as a case of scurvy. Pallor of the skin and mucous

membranes occurs, of course, in nutritional anemia, and pigmentation is frequently found in deficiency of aliments. In the famine in the Netherlands in 1945 such pigmentation was not uncommon, and pigmentation followed by vitiligo-like depigmentation was also seen; the latter usually had the very characteristic depigmentation around the anus such as is found in kwashiorkor.

In this disease greying of the hair is found, and such was also encountered in the Netherlands. By analogy with the fur of rats, deficiency of pantothenic acid or of *p*-aminobenzoic acid might be the cause, but such analogies are very dangerous. In deficiency of aliments a downy hirsutism may appear particularly on the face. 'Staring' hair has been mentioned as being of diagnostic importance in assessing nutriture, but it is very doubtful whether it can be properly appraised even in children; Adamson *et al.* (1945) found it in a tenth of all subjects examined in Newfoundland, but how it was appraised in men over 60 years of age is not mentioned. During our study of the famine in the Netherlands an experienced Dutch pediatrician selected for us a boy with 'staring' hair; he was later presented again to the pediatrician after his hair has been brushed, and another boy not previously selected whose hair was later ruffled was also presented; the 'staring' quality was now found to have passed from one to the other. Hair is easily collected in nutritional surveys, and its chemical analysis might well repay study.

The relation of nutritional disturbances to onychodystrophies has not been adequately investigated. Koilonychia can be caused by deficiency of iron and apparently also by deficiency of calcium (or vitamin D). Atrophic nails, thin and brittle, can be caused by malnutrition; and longitudinal ridges, which may be of no diagnostic significance, can apparently be caused by chronic avitaminosis (White, 1934). Leuconychia has no significance in the assessment of nutriture.

Tongue. The tongue is interesting in that it has been extensively studied in nutritional disorders in man, but hardly at all in lower animals. And the former is being assisted by adequate glossoscopy and by recording through color photographs or tongueprints.

It is well-known that atrophy of the papillae, both filiform and fungiform, can be caused by deficiency of iron, and they will regenerate within a few days after iron therapy; this result is too rapid to be due to an increase in hemoglobin. The smooth tongue produced by deficiency of the hemopoietic factor, as in pernicious anaemia, regenerates quickly when treated with liver preparations, and in this case the filiform papillae reappear first (Frantzell *et al.*, 1945).

The tongue in pellagra has received much attention, particularly from the pen of Kruse in a preliminary report (1942b). His detailed

descriptions of the four acute and five chronic stages of aniacinosis, each with three degrees of intensity, is challenging; and the full report of his observations is awaited. At the time of the preliminary report 5 years ago, in 3 of 15 persons given specific therapy the lesions had "almost completely disappeared"; and this preliminary report, without discussing differential diagnosis, makes the sweeping and unacceptable claim that "From these lines of evidence, glossitis is seen to be a specific sign of pellagra and aniacinosis."

Whereas denudation of the epithelium of the papillae with an active circulation is the generally accepted picture in niacin deficiency, slight denudation with engorged dilated capillaries and vascular stasis with edema is found in deficiency of riboflavin (Sydenstricker, 1941, Kruse *et al.*, 1940). Though no doubt the smooth scarlet and the pebbled magenta tongues are very important and justify color-matching similar to that introduced by Lewis (1929) for the skin, their specificity remains to be proved. But there is no doubt that the epithelial cells of the tongue respond quickly to metabolic disturbances caused by malnutrition, and also regenerate with phenomenal speed when the disturbances are corrected. Further study would be well repaid. It must be remembered, however, that those cells are also very sensitive to trauma: we found in England during the war and in the famine in the Netherlands that an upper denture was the commonest single cause of denudation of the filiform papillae in adults (Sinclair, 1948b); and hot food or spices or excessive smoking (when this was possible in England) will produce bright red fungiform papillae. Fissures of the tongue are also deceptive because they can occur congenitally as in the familiar scrotal tongue.

Gums. Gingivitis is a classical feature of scurvy, and has been regarded almost without question as a specific lesion of deficiency of ascorbic acid. King (1943) and several others have claimed a connection between niacin and Vincent's gingivo-stomatitis, but this has been disputed by several authors.

Unfortunately most of the work on the relation between ascorbic acid and gingivitis will not bear a critical analysis. And seldom in nutritional surveys are reliable statistics regarding the incidence of gingivitis presented. It is usually forgotten that in edentulous persons the incidence of gingivitis is negligible, and therefore data about the presence of teeth must be recorded, since otherwise the apparent incidence of gingivitis will increase and then diminish with age; this elementary fact has usually been overlooked.

Kruse (1942c) has presented a preliminary report based on an examination of the gums macroscopically and biomicroscopically. He believes that "in all respects, therefore, examination of the gums forms a satis-

factory basis for appraisal of vitamin C status . . . [The changes in the gingival tissue] are specific and constant in occurrence. They are present in all states of avitaminosis C: they appear early, persist and reflect its course." Since in the persons examined by Kruse gross manifestations were readily seen in many, it is surprising that in Crandon no macroscopical changes were detected when he had clinical scurvy; it may even be doubted whether deficiency of ascorbic acid produces macroscopical changes in the gums in the absence of other factors such as trauma: constancy in occurrence of gingival changes is certainly doubtful. More important, Kruse's claim of specificity for the gingival lesions he describes cannot be accepted. Redness, swelling, and bleeding of the gums can be produced in monkeys by injections of estrogens (Ziskin, 1937), and in women tend to occur with menstruation and particularly with pregnancy. This has sometimes led to false claims about an increase of deficiency of ascorbic acid in women who are pregnant. Further, there is every reason to believe that the normal gum is pitted rather like orange-peel, and there is no justification for attributing this, as Kruse does, to the presence of atrophy caused by chronic deficiency of ascorbic acid. A group of distinguished nutritional experts who made a survey in Newfoundland (Adamson *et al.*, 1945) believe, with Kruse, that "The most frequent and reliable early clinical signs of vitamin C deficiency are to be seen in the gums." On the basis of macroscopical changes in the gums, they conclude: "One or more obvious signs of either acute or chronic gum involvement occurred in 69% of the population examined. . . . The clinical evidence indicates a high prevalence of a deficiency of ascorbic acid among the people of Newfoundland." Grave exception should be taken to these statements.

Certainly the color of the gums and the presence of swelling and also bleeding under defined conditions should be noted in assessing nutriture. But these signs may not be present in deficiency and most certainly are not pathognomonic of deficiency; and their incidence should be interpreted in association with relevant findings: these include the presence and state (including malposition) of the teeth; mouth breathing, heavy smoking and other forms of trauma; and pregnancy.

Teeth. In lower animals dentine is affected by deficiencies of calcium, phosphorus, vitamin D, and ascorbic acid; enamel is affected by deficiencies of vitamin A and magnesium, and by excess of vitamin A or fluorine. For reasons already mentioned, the presence or absence of teeth and of an upper prosthesis must be recorded, and it is usual to record the incidence of caries; if this is done, the presence of fluorosis should also be noted.

The aetiology of caries cannot here be discussed, but the relevance

of the incidence of caries to an assessment of present nutriture is highly doubtful. The relation of deficiency of fluorine to dental caries is beautifully shown by McClendon's studies (McClendon, 1944; McClendon and Foster, 1945) on the production of caries in rats on fluorine-free diets, and by studies in children of applying sodium fluoride to certain teeth (Knutson and Armstrong, 1945); if caries is recorded, mottling of the enamel should be included.

Mucous Membranes and Mucocutaneous Junctions. The buccal mucosa, the lips and those mucocutaneous junctions that are readily inspected, play a prominent part in assessment of nutriture. Cheilosis is a condition, described by Sebrell and Butler (1938, 1939), in which there is denudation of the epithelium of the lips along the line of closure, most commonly in the centre of the lower lip; the lip becomes abnormally red and may be sore. The condition occurs in deficiency of riboflavin but is not pathognomonic and can be caused by climatic exposure; Machella (1942) showed its nonspecificity, and Cayer *et al.* (1945) found deficiency of niacin and not of riboflavin was related to cheilosis in their patients. It differs, and should be distinguished, from angular stomatitis which consists of ridging and later fissures arising at the mucocutaneous junction at the corner of the mouth. Angular stomatitis also occurs in deficiency of riboflavin but is also not pathognomonic: it can be caused by trauma arising from an ill-fitting upper prosthesis. We use the term mycterosis for a similar condition arising at mucocutaneous junctions: the palpebral fissure, the nares, the external auditory meatus, the prepuce, vulva, and anus. Sydenstricker (1941), Mitra (1943), Stannus (1944) and many others have described it in deficiency of riboflavin, and it is certainly an important sign.

Three forms of stomatitis (acute catarrhal, aphthous, and Mikulicz's) tend to occur in malnutritriture. Buccal denudation along the line of closure of the teeth has been attributed by Sandstead (1943) to deficiency of riboflavin, but this is certainly not a usual cause of this fairly common condition.

Eyes. In lower animals the cornea is affected by deficiencies of vitamin A, riboflavin, tryptophan, lysine, histidine, sodium, and zinc. The conjunctiva is affected by deficiencies of vitamin A, sodium, and perhaps riboflavin; the lens by deficiencies of calcium, riboflavin, and tryptophan; the retina by deficiency of vitamin A; and hemorrhages within the eye bulb occur in deficiency of choline and in hypervitaminosis A. Deficiency of vitamin A affects the lacrimal glands, of sodium the tarsal glands, and of pantothenic acid the Harderian glands. The relevance of these studies to man is doubtful. Deficiencies of the seven nutrients listed above as affecting the cornea all cause corneal vasculari-

zation in the rat; but the cornea of the rat in particular seems to be especially subject to vascularization, and other animals might well be studied.

The full and somewhat melancholy story of corneal vascularization in man cannot here be told. Following the work on rats that started with Day's observations in 1931, Sydenstricker, Kruse and colleagues (Kruse *et al.*, 1940; Sydenstricker *et al.*, 1940) described corneal vascularization in subjects made deficient in riboflavin. The authors regarded conjunctivitis as the primary step in the development of superficial keratitis in ariboflavinosis, and interstitial keratitis also resulted from such deficiency and could involve the whole depth of the cornea; a specific type of glossitis often occurred before other signs of ariboflavinosis were present (Kruse *et al.*, 1940), but later it was asserted that in this deficiency "the earliest and most constant finding is a superficial vascularization of the cornea" (Sydenstricker, 1941). Numerous subsequent papers have used corneal vascularization or circumcorneal injection as specific signs of deficiency of riboflavin; and at the other extreme Vail and Ascher (1942) have suggested that what Sydenstricker termed "corneal vascularization is nothing more than extensive engorgement of the preexisting limbal meshwork" except in cases of obvious corneal disease. Unfortunately two most important questions have been frequently overlooked: what is to be regarded as pathological vascularization of the limbus, and whether pathological vascularization is pathognomonic of deficiency of riboflavin. Everyone will, or should, agree that circumcorneal injection is not pathognomonic of deficiency: it is easily produced by rubbing the eye. The normal limbus, which has been excellently studied by Graves (1934), is supplied throughout its width with capillaries; this might be expected since the limbus is the whole zone in which the sclera and cornea overlap, and the sclera is vascular. In his early papers, however, Sydenstricker (1941) spoke of "the normal avascular zone between the plexus and the sclero-corneal junction"; later he regarded the limbus as vascular. The point is important because if we are to regard the limbus as normally avascular, as Kruse and others do, the incidence of corneal vascularization will be 100%, particularly if some substance such as dionine is used to dilate the temporarily occluded capillaries. It is not surprising, but depressing, to find therefore that in the survey conducted in Newfoundland by several distinguished experts (Adamson *et al.*, 1945), "100% showed some degree of vascularity of the cornea." During the war my own eyes were examined by seven experts in England (including Dr. Sydenstricker) and pronounced normal; after flying the Atlantic they were examined by seven experts in the U.S. and Canada and all seven said I had ariboflavinosis (according to Mr. Basil Graves, I have con-

centric collaterals as described by him (1934) in the normal limbus and by Vail and Ascher (1942) as occurring as a result of some "provoking cause"); but I had then taken riboflavin daily for three years and drunk beer, in moderation, for twenty-six years. This difference in interpretation is confusing. Obviously the extreme inner limit of the limbus must be defined: as mentioned elsewhere, polarized light assists this; and dionine helps in revealing the capillaries. But if capillaries overstep the limbus and invade the cornea superficially and fairly symmetrically and usually in both eyes, the condition may be due to deficiency of riboflavin or it may not. The dietary history, the presence of other signs of possible ariboflavinosis (so admirably described by Sydenstricker in various publications), the level of riboflavin in leucocytes or other tissues, and the response to specific and controlled therapy, these will decide the etiology of the pathological lesion and without at least one of them the decision cannot be made.

Since deficiency of certain amino acids has been found to produce corneal vascularization in the rat (Totter and Day, 1942; Albanese *et al.*, 1943; Sydenstricker *et al.*, 1946) we examined 155 persons attending a "polyclinic" for malnourished patients during the famine in the Netherlands; the incidence of corneal vascularisation was 7%, which is higher than that found in the same age groups in England.

Xerosis and keratomalacia are classical signs of deficiency of vitamin A (Pillat, 1931), and vascularization of the substantia propria occurs probably as a secondary phenomenon. It is customary to regard the changes in the corneal epithelial cells as the direct effect of deficiency of vitamin A upon them, but it is possible that part at least of the changes may be produced by deficient secretion of the ocular glands: in Sjögren's syndrome, keratitis occurs. Xerosis and keratomalacia are late changes in deficiency of vitamin A, and a characteristic feature is the painlessness of the condition.

Changes in the conjunctiva in nutritional deficiencies have achieved much prominence. Marked conjunctivitis, such as occurs in snow blindness, has been attributed to deficiency of riboflavin; and it certainly appears from the work of Sydenstricker that such deficiency is one cause of conjunctivitis. More important is deficiency of vitamin A. Kruse (1941) has presented a preliminary report of the conjunctival manifestations of avitaminosis A, which he found in 99% of the subjects he examined. Unfortunately no distinction is made in his description of conjunctival spots between pingueculae and true Bitot's spots. Pingueculae, of course, are caused by degenerative changes in the fibrous tissue in the substantia propria beneath the epithelium which is normal; Bitot's spots are quite different in appearance and are epithelial in origin. There

is no evidence that pingueculae or pterygia are nutritional in origin. Bitot's spots, which are very rare in temperate climates, can no doubt be caused by deficiency of vitamin A, and this is probably their commonest cause; but we have had the opportunity of studying cases that were not, and probably had not been, accompanied by such deficiency. Kruse suggests that xerosis conjunctivae probably precedes night blindness as an early sign of avitaminosis A. We have had the opportunity of repeatedly examining with a biomicroscope the eyes of volunteers placed upon diets deficient in carotenoids and vitamin A in two separate experiments: in neither experiment was any change in the conjunctival epithelium detected in any subject, and in both experiments the final rod threshold of the dark-adapted eye of some of the subjects rose significantly and reverted to normal after therapy with halibut-liver oil.

Changes in the lens occur in deficiencies of calcium (Meyer and Meyer, 1944), riboflavin (Day *et al.*, 1938) and tryptophan (Totter and Day, 1942; Buschke, 1943) in lower animals. In man, insufficient attention has been paid to structural changes in the lens in nutritional deficiencies, and such changes might well be important.

Glands. No discussion of nutritional deficiencies as affecting glands is possible here. Changes in the sebaceous glands occur in deficiencies of vitamin A and of riboflavin. Apart from the thyroid gland, in which epithelial hyperplasia is apparently produced by deficiency of iodine or excess of fluorine, few other glands are conveniently included in the assessment of nutriture.

Nervous System. Possible changes in the nervous system in deficiencies of thiamine, riboflavin, niacin, and copper cannot here be discussed, nor can the neurological conditions that arose as a result of wartime dietary restrictions; these have been admirably discussed by Grande and Peraita (1941), Denny-Brown (1947), and Spillane (1947). In a few experiments in which human volunteers have been placed upon a diet deficient in thiamine, polyneuritis has definitely been produced, but the accompanying lesion, presumably morphological as well as biochemical, has taken a very long time to respond to specific therapy (Williams *et al.*, 1943). Retrobulbar neuritis has been mentioned elsewhere in this article; the precise nutritional causes of spinal ataxia, spastic ataxia, spastic paraplegia, and "burning feet" are obscure but deficiency of the vitamin B_2 complex seems to be concerned.

In a rapid assessment of nutriture, the elicitation of knee- and ankle-jerks, of vibratory sense, and of muscular weakness (as, for instance, elicited by the squatting test) are of interest; but it is obvious that the presence of one or more of these signs must be regarded as an indication for a fuller examination. The Newfoundland survey (Adamson *et al.*,

1945) found one or more of these signs present in 6.8% of the population examined and "in view of this and of the numerous unsolicited complaints referable to the nervous system, it is likely that the thiamine status of a considerable proportion of the population is unsatisfactory"; "likely" is far too strong a word, and the statement elsewhere that "clinical signs which result from a lack of thiamine were encountered in 6.8% of the persons examined" is highly misleading.

Connective Tissue, Muscle, Bone and Cartilage. The relation of ascorbic acid to the differentiation of mesenchymal cells to form fibroblasts with production of collagen, and the relation of ascorbic acid and of calcium to the formation of intercellular cement substance, are complicated and mainly relevant here in consideration of the defects in wound healing that occur in deficiency of ascorbic acid.

Cartilage and bone are specialized connective tissues which are affected by deficiencies of calcium, phosphorus, vitamins D and A, and manganese. Deficiency of vitamin A slows endochondral bone formation, decreasing osteoblastic activity, and growth of cartilage cells; and if in excess vitamin A stimulates bone growth. In deficiency of ascorbic acid cartilage grows normally but fibroblasts fail to form osteoid. In deficiency of calcium or phosphorus or vitamin D, or in the presence of abnormal dietary ratios of calcium and phosphorus, there is defective deposition of these two elements in growing cartilage and osteoid.

The clinical diagnosis of active rickets is very difficult except in gross forms. At about 3 months of age, craniotabes is the most useful sign; at about 6 months, enlargement of the chosto-chondral junctions; towards the end of the first year, enlargement of the epiphyses at the wrist; and after the eighteenth month, delayed closure of the anterior fontanelle should be considered. The question of diagnosis of rickets is considered in the excellent review of Eliot and Park (1943) and in the report of the British Paediatric Association (1944); the latter shows the great variability in the clinical assessment of the incidence. The earliest diagnosis can be made by chemical methods; next by radiology; last by a clinical examination. By this examination alone it may be impossible to differentiate between rickets that is active or has been cured for months or even years. Radiological estimations of skeletal maturity and skeletal mineralization may prove valuable; they are discussed in the publications of Mack whose extensive studies will be very helpful (Mack and Smith, 1939).

Heart muscle in lower animals is affected by deficiencies of thiamine, potassium, and vitamin E; and deficiency of vitamin E affects skeletal muscle. But there is no clear evidence that vitamin E is necessary in man: there is no valid reason for believing it prevents abortion in women

or sterility in men or muscular dystrophies; and the latest paper by Vogelsang and colleagues (1947) wastes further paper and time. Muscle tenderness is a useful sign, since it appears relatively early in deficiency of thiamine. Winged scapulae and other postural defects are of very doubtful value.

Respiratory System. In deficiency of vitamin A, the ciliated columnar cells of the trachea and bronchi become replaced by keratinizing epithelium. This may block bronchi les, causing atelectasis. Dr. Jonxis of Rotterdam found that this occurred during the famine in the Netherlands, and a radiological examination of the chest was useful in revealing it.

Circulatory System. Heart muscle and changes in blood pressure and pulse rate have been mentioned elsewhere.

Nutritional edema may be divided into three types (Sinclair, 1948a). First, there is wet beriberi caused by deficiency of thiamine and a diet relatively high in carbohydrate. Secondly, there is hypoproteinemic edema caused by a lowered concentration of protein, particularly albumin, in the plasma with a consequent fall in osmotic pressure; but deficiency of aliments, such as occurred in concentration camps during the war, can lead to gross emaciation and very low values for plasma protein without edema. Thirdly, there is what may reasonably be called "famine edema" since it arises in persons who subsist on diets deficient in aliments; the persons are not necessarily emaciated and values for plasma protein are usually within the statistically normal range. The diagnosis of this third type in its mild forms is very difficult, since the person may appear to be well nourished: we have seen in Civil Internment Camps in Germany many males high in mesomorphy and low in gynandromorphy who at a glance appear well nourished and yet show pitting nutritional edema. The diagnosis must be made on the dietary history, medical history (especially nycturia) and other supporting signs (bradycardia, low blood pressure, acrocyanosis, etc.); and it is obvious that nonnutritional causes must be eliminated.

In erythropoiesis, various nutrients are probably concerned: certain essential amino acids, iron, copper, cobalt, pteroylglutamic acid, pyridoxine, riboflavin, and niacin. Deficiency of iron, copper, or pyridoxine causes microcytic hypochromic anemia. Pallor of the skin, nail bed, and mucous membranes is very deceptive in many cases; in deficiency of aliments there is a curious greyish-lemon tinge of the skin.

Gastrointestinal System. This is not normally examined in assessment of nutriture, although obviously abdominal signs will be sought if there is special indication. The very extensive work that has been done on damage to the liver by nutritional deficiency cannot be discussed here.

Genitourinary System. Testicular atrophy occurs in many nutritional deficiencies, and deficiency of arginine appears to cause decreased spermatogenesis in man (Holt *et al.*, 1942).

Amenorrhea can be caused by deficiency of aliments, but circumstances that accompany undernourishment may cause psychical amenorrhea: we noted during the famine in the Netherlands that some women who had complained of this sign started to menstruate again on or about the day of liberation.

VII. Discussion—Summary

The *nutriture* of the body depends upon the *nutrition* of the cells of the body, and this process involves *aliments* (which include substances that are used by the body for the production of energy, such as carbohydrate, fat and protein), *nutrients* (which include essential amino acids, essential fatty acids, vitamins, provitamins and compounds of certain essential elements), oxygen and water. Nutrition may also be affected by the accumulation of metabolic products or the presence of toxic substances, or by the action of physical agents such as heat or ultraviolet radiations. *Health* depends upon the degree of nutriture and upon the presence or absence of nonnutritional disease; the *physical state* of the body depends upon health and upon the presence or absence of structural defects which may or may not have been caused by past malnutriture. There are three important *properties of nutriture: quality* expresses the kinds or types of nutritional deficiencies; the *degree* of nutriture may arbitrarily be classified into five categories with death from surfeit or deficiency as limits; the *duration* of nutriture describes the time during which the state has existed. The acuteness or chronicity of the process causing the state is determined by the rate of change of degree with respect to time. The *development of malnutriture* usually occurs in four stages: inadequate ingestion, decreased bodily reserves, impaired function, anatomical lesions. Dietary, chemical, functional, and clinical methods may therefore be used; since growth and body weight are affected by nutriture, somatometric methods are also relevant. With any of these methods, the *concept of normality* is involved. Two definitions of normal, one based on physiological and the other on statistical concepts, are used: the condition of the body, or of some part or function of the body, is (physiologically) normal when it allows the usual functions to be adequately performed in the usual environment; a value of a variate is statistically normal when it does not differ from the mean by more than the standard deviation.

Dietary methods usually cannot alone be used to assess nutriture. *Chemical methods* are very valuable, and include estimations of substances

in tissues, blood, or urine. The composition of tissues alters before structural changes occur through malnutriture; estimations of vitamins of the B complex and of ascorbic acid in tissues such as leucocytes are particularly useful. Compounds in plasma vary in their behavior: some, such as carotenoids and ascorbic acid, tend to reflect the immediate past intake; others, such as niacin and riboflavin and usually protein, remain at concentrations that are within the statistically normal range even though clinical malnutriture may be present. In these cases a single estimation that falls within the statistically normal range gives little information. Estimations upon urine, with or without test doses, have not yet proved valuable for assessing nutriture.

The most useful of the *functional methods* is the measurement of the rod threshold of the dark-adapted eye provided a high value is tested again after therapy with vitamin A. The measurement of nerve accommodation may prove to be of similar value in detecting deficiency of thiamine. Measurements of the fragility of capillaries in the skin or gums are not helpful in assessing deficiency of ascorbic acid. Measurements of strength with dynamometers serve no useful purpose in nutritional surveys, and in these it is usually impracticable to include tests of physical fitness, of capacity for physical work, or of fatigue; a reliable test of endurance should be useful particularly in longitudinal studies.

Somatometric methods, such as the use of sex-age-height-weight tables or the calculation of various indices based on body measurements, are by themselves useless in the cross-sectional assessment of nutriture in an individual. A large number of nonnutritional factors condition growth, and malnutrition does not necessarily retard growth or diminish adult weight. Further, the optimum rate of growth of children is not necessarily the maximum. Even longitudinal studies of growth or of adult body weight are not simple. Measurement of the thickness of skin and subcutaneous tissue over the arm is useful in nutritional surveys.

Clinical methods are essential in the proper assessment of nutriture. The general assessment by inspection is usually concerned mainly with physical state, is strongly influenced by body size, and is found not to be reproducible. Symptoms, though subjective, should be recorded. The interpretation of clinical signs is difficult and very often erroneous; almost all the early signs are unspecific. Artifacts or the results of other nonnutritional factors may be misleading, such as unkempt hair, a smooth tongue caused by an upper denture, cracked lips or chapped skin caused by exposure, sun burn or snow blindness, bug-bites or "vagabond's disease." The differential diagnosis of advanced nutritional disease is usually relatively easy, although mistakes, as between pellagra and lupus erythematosus for example, may be made; but the appreciation of the

existence and cause of early departures from normality is difficult. Thorough familiarity with the normal is needed, and this is assisted by various aids to the examination such as the biomicroscope. The tissues that are most often affected structurally by malnutriture are the epidermis, and the cornea.

The *assessment of nutriture* is a diagnosis and, like clinical diagnosis, uses various disciplines. The combination of different methods, such as are mentioned above, is essential. These methods should, whenever possible, be quantitative and possess both precision and specificity so that the facts obtained are adequately described and are reproducible; they can be interpreted anew or reassessed as knowledge advances. The facts should be objective; the interpretation of the facts is subjective. Measurements, like those made by chemical methods, are therefore particularly valuable. Clinical methods are unfortunately subjective to a large degree, and the criteria adopted by the examiner inevitably alter: the standard of normality tends to be affected by what is commonly found in the particular population under examination, and even by what the examiner expects to find. The assessment of deviations from the normal demands a knowledge of the range of normality, and of the factors that enlarge or diminish it. Unfortunately, the study of the relevant parts of the physiology, morphology and chemistry of man has not advanced far; man is a neglected animal and the variations that are found and the causes of these should be more carefully studied. It is obvious that if a nutritional variate is measured in two populations of different nutritures, one normal and one poor, and if the distribution of the variate in each is defined by an approximately Gaussian curve, the two curves are likely to overlap considerably. A significant difference between the means of the values of the variate in the two populations may be found, and this is important in comparing the nutritures of the two populations. But since the values of the variate in the individuals overlap, a single observation in one individual may not give much relevant information: the value may be in the upper range of the deficient group or in the lower range of the normal group. Since a variate has a probability function, our statistical definition of normality would most satisfactorily be in terms of probability; but for practical convenience a less satisfactory definition in terms of the standard deviation has been adopted, although the distribution of the values of the variate may not be approximately Gaussian. Such unsatisfactory practices must be adopted until we know more about the causes of variation and the values of variates at which abnormality, in terms of the physiological definition of normal, arises. For instance, let us assume that vitamin A is measured in the serum of a large number of apyrexial adults all of whom have nor-

mal nutriture regarding this vitamin, and that the mean is 100 arbitrary units and the standard deviation 33. Then, according to our definition of statistical abnormality, individuals having values below 67 or above 133 units would be abnormal. This of course does not mean that they have malnutriture regarding vitamin A: we have already said that they have normal nutriture regarding this vitamin. Similarly we might say that the values falling below 34 or above 166 units (that is, outside the limits of twice the standard deviation from the mean) are extremely abnormal. If the distribution of the values is Gaussian, about 84% of the values will not be abnormally low, about $13\frac{1}{2}$% of the values will be abnormally low but not extremely abnormal, and about $2\frac{1}{4}$% of the values will be in the lowest category of abnormality. If however it were found that a value below, say, 30 units maintained for a long time was invariably accompanied by a significantly increased rod threshold of the dark-adapted eye whereas a value not less than 30 units was similarly not accompanied by a significant increase in rod threshold, and if a significant increase in rod threshold were the first indication of poor nutriture regarding vitamin A, then we would be justified in taking a value below 30 units as indicating abnormality in the physiological sense. In practice we have not the requisite information to analyse variates, such as chemical levels in blood or dietary requirements of aliments and nutrients, in terms of either the physiological or the statistical definition of normality. Despite this, arbitrary limits must be adopted as a practical convenience. Those used, for chemical and for two functional estimations, by the Oxford Nutrition Survey early in its work are listed in Table I (Sinclair, 1947a, corrected). Data have now been collected that will shortly make it possible to amend these limits and make them less arbitrary.

Nutritionists, school medical officers and others concerned with the public health often try to assess general nutriture in individuals. The attempt smacks of the alchemist's search for the philosopher's stone. Spearman in his studies of intelligence showed that there was a general factor (g) and specific factors (s); it is sometimes thought that general nutriture (which we may call N) can similarly be rated in individuals. The general nutriture of the body is the sum of the specific nutritures (N_s) of the cells of the body, N_s depending upon the factors (such as nutrients) already discussed as affecting the nutrition of cells. N is a statistical concept and can be assessed only in terms of its effect on health; this can only be measured quantitatively in terms of probability of death (which is zero health). Whereas we cannot therefore measure N, or for example say whether a child with severe active rickets has worse general nutriture than one with mild xerophthalmia and mild scurvy, except in terms of probability of death occurring if the state is continued,

TABLE I

Arbitrary Standards of Normality Used by the Oxford Nutrition Survey

	Estimated on	Units/100 ml. or 100 g.	Abnormal below	Extreme abnormality below
Hemoglobin:				
≮15 years, male	B	g.	13.85	12.05
≮15 years, female	B	g.	12.50	10.70
7–14 years	B	g.	12.05	10.25
2–6 years	B	g.	11.15	9 35
0–1 years	B	g.	10.25	8.45
Erythrocrit:				
≮15 years, male	B	%	41	36
≮15 years, female	B	%	37	32
7–14 years	B	%	36	30
2–6 years	B	%	33	28
0–1 years	B	%	30	25
Leucocrit, low	B	%	0.5	0.3
high	B	%	1.0[1]	1.5[1]
Protein, total	S	g.	6.1	5.3
Albumin	S	g.	4.0	3.2
Vitamin A (adults)	S	I.U.	70	30
Carotenoids	S	γ	100	50
Phosphatase (adults)	S	K.-A. units	10[1]	16[1]
Thiamine hydrochloride	B	γ	4.5	2.0
Pyruvic acid	B	mg.	1.6[1]	2.0[1]
Niacin	B	γ	400	200
Riboflavin	B	γ	20	12
	L	γ	400	200
Ascorbic acid	B	mg.	0.50	0.10
	L	mg.	20	10
Fasting urine (per hour):				
Thiamine hydrochloride	U	γ	7.50	2.51
N'-Methylnicotinamide	U	γ	95.0	45.1
Riboflavin	U	γ	30.0	10.1
Ascorbic acid	U	mg.	0.46	0.15
Rod threshold	Dark-adapted eye	log. μmL.	2.1[1]	2.5[1]
Capillary fragility	Skin of forearm	Petechiae per sq. in.	10[1]	20[1]

B = Whole blood
S = Serum
L = Leucocyte-platelet layer
U = Urine

[1] Abnormal and extremely abnormal values *not less than* these limits.

we can attempt to assess specific nutriture regarding aliments or nutrients by the methods discussed in this article. But *N* is not merely the sum of the specific nutritures that we can assess: just as the experienced clinician can tell that a child is very ill without the aid of a cachometer, so the experienced nutritional observer on the basis of his physical examination can assess the person's nutriture partly by taking into account the presence and combination of the signs discussed above and partly by an ill-defined belief that the person has a particular degree of nutriture. This assessment by the physical examination alone differs from the rapid general assessment by inspection because the former is based on the examination of particular systems (skin, eyes, etc.) whereas the latter is an assessment mainly of physical state. A rapid nutritional survey should include a medical history and a clinical examination with the symptoms and signs being recorded in three grades of abnormality, "mild," "marked," or "severe"; then a tentative clinical diagnosis and a provisional assessment of general nutriture should be made. On the basis of these and of the results of chemical, functional and dietary methods the final diagnosis and assessment of *N* are made. It must be clearly understood that this assessment of *N* is relative only: the more detailed and extensive the methods employed in the assessment, the lower will this be rated. If, for example, the methods we happened to be using gave no information about mild deficiency of ascorbic acid, we should tend to class the individuals as having normal nutriture regarding this nutrient; but if estimations of the nutrient in blood or plasma or leucocytes are included, the assessed state cannot be improved and will probably be lowered. Therefore if comparisons between the general nutritures of individuals or of populations are being made, the methods included must be the same. These methods, which have been discussed in this article, are summarized in Table II.

It is evident therefore that the assessment of nutriture in populations is not, as some seem to think, a matter of training a girl for a couple of days before sending her into the wilds with a box of forms and a slit-lamp microscope; it is a detailed undertaking for a team of workers experienced in different disciplines. The cross-sectional assessment in a single individual is the hardest task, the comparison of two populations by cross-sectional assessments being less difficult. Longitudinal studies are much simpler, whether they follow the nutritures of single individuals or of populations; and if controlled therapeutic tests can be included in either of these, the most satisfactory position is reached. The problem however still remains complex: an increase in the amounts of ascorbic acid in the bloods of the group treated with this nutrient may imply no alteration in the nutriture of the individuals in the group.

TABLE II

Methods for Use in Rapid Assessments of Nutriture in Populations

Primary. A clinical examination, a dietary history and a medical history are *essential.* The other methods placed in the Primary group are *valuable.*

Secondary. The methods in this category are either (a) valuable but need special apparatus or techniques, or (b) simple to perform but need further research for their interpretation or are otherwise of doubtful value.

Tertiary. The methods in this category have no proved value as yet for assessing nutriture in cross-sectional studies.

B indicates determinations on whole blood, L on leucocytes, S on serum or plasma.

Primary	Secondary	Tertiary
Dietary Dietary history Family inventory and purchase method.	(a) Individual method	
Chemical Hemoglobin (B) Erythrocrit (or erythrocyte count) (B) Protein without fractionation (S) Ascorbic acid (B) Vitamin A and carotenoids (S) Phosphatase in children (S)	(a) Pyruvate (B) (a) Protein with fractionation (S) (a) Ascorbic acid (L) (a) Vitamins of the B complex (L) (b) Phosphatase in adults (S) (b) Fasting urinary excretion tests or load tests of water-soluble vitamins	Minerals (B, S) Prothrombase clotting-time (B) Vitamins of the B complex (B) Saturation-tests of water-soluble vitamins
Functional	(a) Rod threshold of dark-adapted eye (b) Endurance tests	Visual acuity Nerve accommodation Ergometric tests Psychometric tests Electrocardiograms
Somatometric Weight Height Thickness of skin and subcutaneous tissue	(b) Sitting-height	Other somatometric determinations
Clinical Examination of skin, eyes, etc. Medical history Blood pressure	(b) Pulse-rate (a) X-ray of hand and wrist in children	X-ray of hand and wrist in adults Capillary fragility Gum fragility

There is no easy method of evaluating *N*, although relevant observations and estimations of deceitfully enticing simplicity may be made. If we may allow ourselves the luxury of a metaphor, we may conclude that through the wilderness of human nutrition there run many paths, some twisted, many beset with snares, and all thorny; the only one leading straight forward is the path of scientific method which is laid by the critical accumulation and sifting of orderly truths, and this path is largely untrodden.

In this article I have drawn upon experience gained by the Oxford Nutrition Survey. I am particularly indebted to two former members of its staff, Mr. B. B. Lloyd and Mr. M. C. K. Tweedie, for their helpful suggestions.

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Vitamins in Microorganisms—Distribution and Quantitative Synthesis

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CONTENTS

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I. INTRODUCTION

Within the past decade our knowledge regarding the existence of new nutritional factors and the manner in which these and earlier recognized vitamins function in metabolism has been greatly extended. Conspicuous as a result of this progress has been the growing realization that these substances have a general rather than a particular significance in biology. Consequently, nutrition studies irrespective of whether they are concerned with higher plants, animals, or fungi have been integrated and share common ground and common objectives. Particularly striking, for example, has been the parallelism between factors essential in animal nutrition and those required for the growth of microorganisms. This similarity has provided an excellent means of investigating these substances since microorganisms have well-defined and often simple requirements and are especially suitable for the examination of isolated biochemical reactions. Actually, the remarkable success which has attended their application has made microorganisms the tool of choice for elucidating the role of vitamins in metabolism. Based on contributions to our knowledge of the structure and function of biotin, pyridoxine, pantothenic acid, folic acid, and *p*-aminobenzoic acid as well as other nutrilites, microbiology is certain to occupy a pivotal position in furthering our understanding of essential nutritional factors.

The most commonly applied and productive approaches to nutritional problems employing lower fungi have involved one of the following: (a) determination of the growth requirements of various species and their derived mutants, (b) determination of the coenzyme requirements of certain enzymes, (c) metabolite-antimetabolite relationships, and (d) the ability of certain species to utilize vitamin precursors and vitamin analogues. Another approach which should provide at least supplemental information relative to the mechanism of vitamin action is that offered by the study of quantitative biosynthesis of vitamins under a wide variety of experimental conditions. Such studies have been initiated only recently and represent a natural progression from earlier

qualitative investigations in which synthesis was assumed when growth occurred in the absence of an external supply of the factor under study. The examination of synthetic abilities, while not developed systematically, has been extended to numerous species. The influence of certain physical and chemical factors upon the degree of activity has been ascertained, including interesting observations linking inorganic elements with vitamin formation. Evidence also has accumulated to show that vitamins not only are synthesized but are absorbed, excreted, and destroyed by microorganisms, the rate and extent of these activities varying with the species and cultural conditions. Needless to say, much further work will be necessary to determine whether such behavior is relevant to either the requirements or the specific functions of these factors for various species.

Aside from theoretical considerations, the elaboration of vitamins by microorganisms has become of increasing commercial importance. Certain species capable of producing extraordinary amounts of riboflavin, pantothenic acid, biotin, and folic acid have been discovered and others have been described which acquire and store substantial quantities of thiamine and niacin. Some of these have been put to industrial use and others appear to be worthy of consideration in this respect. Other than microbiological processes designed specifically for vitamin production, the biosynthesis which takes place, for example, in commercial butanol, ethanol, and penicillin fermentations contributes to the value of the residues recovered as feed adjuncts. A better understanding of those factors which control vitamin synthesis in such fermentations would not only make for greater uniformity of feed by-products but also offer the possibility of improving the potency of the more valuable constituents.

In the present review an attempt has been made to correlate available information on the content and synthesis of vitamins by microorganisms. For the most part, only quantitative data were considered. However, it should be emphasized that some of the data, particularly on the content of cells, were collected under diverse experimental conditions and therefore are not strictly comparable. They are intended primarily to illustrate the range of potencies to be expected rather than to provide a basis for delineating species or genera.

Intestinal synthesis and the like in which mixed cultures are known to be operative have been covered previously (Najjar and Barrett, 1945) and thus have been omitted from this discussion. Likewise, the occurrence in, and elaboration of unidentified animal growth factors by microorganisms, although well-recognized and of decided current interest, have not been included because of space limitations and the fragmentary nature of quantitative data.

II. Thiamine

1. Content of Microorganisms

Listed in Table I are the thiamine potencies of various bacteria, yeasts, and molds. Within certain species, e.g., *Torula utilis*, there is considerable variation in content which in most instances is attributable to the use of different media and cultural conditions. Nevertheless, the

TABLE I

Thiamine Content of Microorganisms

	Dry cells, µg./g.	References
Bacteria		
Aerobacter aerogenes	11–15	315
Azotobacter chroococcum	33–96	149, 201
Clostridium butylicum	9.3	315
Phytomonas tumefaciens	12	180
Propionibacterium pentosaceum	0.4–6.2	271
Proteus vulgaris	21	315
Pseudomonas fluorescens	26	315
Serratia marcescens	27	315
Unidentified isolate from rat caecum	48	1
Yeasts		
Candida arborea	31.3–33.1	3
Endomyces vernalis	15–34	212, 322
Hansenula suaveolens	8.5	132
Mycotorula lipolytica	5.3	132
Oidium lactis	20.1–29.0	3
Saccharomyces carlsbergensis	31	322
Saccharomyces ellipsoides	38	322
Saccharomyces cerevisiae	29–90	3, 211, 272, 322
Saccharomyces logos	7.5–30	212
Torula utilis	6.2–52.8	71, 132, 156, 157, 205, 248, 272, 300, 314
Willia anomala	10–30	212
Zygosaccharomyces pini	39	322
Commercial bakers' yeast	9–40	176, 177, 211, 322
Commercial bakers' yeast (enriched)	650–750	176, 177
Commercial foil yeast	80–150	176, 177
Commercial brewers' yeast	50–360	39, 71, 172, 266
Molds		
Aspergillus oryzae	18	251
Fusarium lini Bolley	20	326
Fusarium graminarum	5	326
Penicillium notatum	6.9	200
Penicillium chrysogenum	2.6	200

three groups of organisms exhibit the same range of values particularly when grown on essentially thiamine free substrates. No species is an unusually rich source of thiamine and only *Propionibacterium*, partially deficient for thiamine, is of exceptionally low potency.

As discussed in detail below, the thiamine which occurs in microorganisms arises from (a) total synthesis, (b) absorption from the medium, and (c) partial synthesis involving precursors. Absorption and partial synthesis are the more important from a quantitative viewpoint although other factors significantly influence synthesis and cell potencies.

2. *Microbial Synthesis*

Bacteria were early recognized as a source of B vitamins. Damon (1923) showed that vitamin B was elaborated by Pfeiffer's bacillus, *Mycobacterium phlei*, and *Mycobacterium malleri*. A few years later Scheunert and Schieblich (1927) demonstrated that *Bacillus vulgatus* cells, grown on a medium devoid of vitamin B, promoted the growth of pigeons maintained on a B-deficient diet. *Bacillus mycoides* and *Bacillus mesentericus* contained less of the growth-promoting principle than *B. vulgatus*. The ability of *B. vulgatus* to synthesize thiamine was confirmed subsequently by Guha (1932) and by Burkholder and McVeigh (1942).

Although many species remain to be surveyed, it is now assumed that most bacteria are capable of thiamine biosynthesis to meet their metabolic requirements. When cultivated on carbohydrate-nutrient salt mixtures, thiamine has been quantitatively determined in the cells of *Rhizobium trifolii* (West and Wilson, 1938), *Phytomonas tumefaciens* (McIntire *et al.*, 1941), *Azotobacter chroococcum*) (Lee and Burris, 1943) and in various intestinal bacteria (Burkholder and McVeigh, 1942). From more complex but thiamine-free substrates, thiamine has been demonstrated in the cells of *Aerobacter aerogenes*, *Serratia marcescens*, *Pseudomonas fluorescens*, *Proteus vulgaris*, *Clostridium butylicum* (Thompson, 1942), *Escherichia coli*, *Bacterium bifudum* (Reichelt, 1941), *Mycobacterium tuberculosis* (Lutz, 1947), and in various actinomycetes (Herrick and Alexopoulos, 1943). Another notable example of synthesis is that effected from wholly inorganic medium constituents by *Thiobacillus thiooxidans* (O'Kane, 1942).

Thiamine formation by bacteria varies with the strain of organism and its cultural history in addition to the medium and culture conditions. That bacterial cells which normally are heterotrophic for thiamine can be trained to synthesize their needs is indicated from the data of Silverman and Werkman (1939) with *Propionibacterium pentosaceum*. By acclimatization in the absence of thiamine cells increased in content

from 0.4 to 6.25 μg./g. Presumably because they synthesize thiamine, other species of *Propionibacterium*, such as *P. freudenreichii*, grow equally well in its presence or absence (Tatum *et al.*, 1936). Quantitative differences among strains of *Mycobacterium tuberculosis* have also been observed by Lutz (1947) who found that human, avian, bovine, and BCG strains produced respectively, 19.45, 14.75, 10.04, and 7.75 μg. thiamine/g. dry cells.

Burkholder and McVeigh (1942) report appreciably higher thiamine values for intestinal bacteria than other investigators, in some instances for the same species. On a wet cell basis their figures for synthesis were as follows: *Escherichia coli* 115, *Bacillus vulgatus* 72, *Proteus vulgaris* 95, *Aerobacter aerogenes* 43, *Alkaligenes fecalis* 132, and *Bacillus mesentericus* 53 μg./g. Total synthesis (cells and medium) for *A. aerogenes* and *P. vulgaris* were reported by Thompson to be only 19.9–26 and 21–23 μg./g. dry cells, respectively (Thompson, 1942). Strain variation as well as culture conditions and the period of incubation may partially explain these differences. Genung and Lee (1944) reported that the thiamine content of the medium influenced the production of this vitamin by certain strains of *Escherichia coli*. This is a singular case since, generally within the limits of concentration usually encountered, the presence of a vitamin in the medium does not affect its synthesis.

Although relatively few studies have been carried out on the coupling of thiamine intermediates by bacteria, the work of Knight (1937) with *Staphylococcus aureus* demonstrates that this property is not restricted to yeasts and fungi which are discussed later. Likewise, certain bacteria tend to retain synthesized thiamine and may absorb additional quantities from solution. Scheunert and Schieblich (1927) noted that cells of *Bacillus vulgatus* possessed vitamin B activity whereas the cell-free medium was inactive. Similarly, with an unidentified organism isolated from rat caecal contents Abdel-Salaam and Leong (1938) showed that cells increased in thiamine content during the early hours of incubation and attained highest potencies at 24 hours. Thereafter, during a 9-day incubation period the level in the cells gradually decreased. It is of interest that these investigators offered the explanation confirmed by later studies (Hochberg *et al.*, 1945; Parsons *et al.*, 1945) that, due to the retentive ability of intestinal organisms for the vitamin B which they produce, no benefit is derived by the host maintained on a deficient diet.

In molasses medium Lee and Burris (1943) found that *Azotobacter chroococcum* cells became approximately three times as rich in thiamine as when propagated in sucrose medium. Since all the thiamine was concentrated in the cells it is probable that absorption from the molasses medium accounted for the difference in potency. However, not all

bacteria completely retain synthesized thiamine. Thompson (1942) found from about 10 to 60% of thiamine produced by various bacteria to be excreted into the medium within 24 hours.

That yeasts are capable of total synthesis of thiamine from carbohydrate-nutrient salts media was disclosed by Scheunert *et al.* (1939). They noted that *Torula utilis* contained thiamine after eleven continuous transfers in thiamine-free synthetic medium. Elaboration of thiamine on synthetic media has been observed as well with *Saccharomyces logos*, *Endomyces vernalis*, and *Willia anomala* (Pavcek *et al.*, 1938) and with *Saccharomyces cerevisiae* (Van Lanen *et al.*, 1942). On thiamine-free substrates, however, some species of yeasts have greater synthetic capacities than others. Livshits (1941) found *Torula utilis* and a strain of commercial brewers' yeast more productive than *Saccharomyces logos*, bakers' or distillers' yeast; whereas of several types valuated by Pavcek *et al.* (1938), *Endomyces vernalis* yielded approximately double that obtained with other species. Some increase in the synthetic ability of yeasts might result, too, from continuous subculturing in the absence of thiamine according to Leonian and Lilly (1943).

From the reports of Fink and Just (1941a, 1942a), Lewis *et al.* (1944) and Odintsova (1941), it is clear that anaerobic conditions are more conducive than aerobic to thiamine production. The higher potencies of anerobic cells cannot be ascribed merely to enrichment through absorption since cells maintained anaerobically in the absence of thiamine also increase in potency (Maizel, 1946). Similarly Odintsova (1941) obtained values of 10 and 20 μg./g. for *Torula utilis* grown aerobically and anaerobically respectively, in synthetic medium.

Yeasts grown on wood sugars and especially wood sugar stillage (Kurth and Cheldelin, 1946) are considerably lower in potency than those propagated on molasses perhaps owing either to enrichment by absorption of thiamine from molasses or to an inhibition of synthesis by substances contained in wood hydrolyzates. A relatively low incubation temperature and a pH of approximately 4.3 were observed by Pavcek *et al.* to enhance synthesis (1937, 1938). In media deficient in iron, thiamine production by *Torula utilis* was increased appreciably (Lewis, 1944).

Fluoride and compounds which react with carbonyl groups such as cyanide, sulfite, semicarbazide, and hydroxylamine reduced synthesis (Eppright and Williams, 1946). Pyrithiamine which interfers with the action of thiamine does not markedly influence its formation by microorganisms (Woolley and White, 1943).

Most lower fungi are autotrophic for thiamine, as is demonstrated by the studies of Fawns and Jung (1933) with *Aspergillus niger*, Scheunert and Schieblich (1936) and Sakurai (1940) with *Aspergillus oryzae*,

Penicillium glaucum, and *Mucor racemosus*, Carpenter and Friedlander (1942) with various fungi, and Vinson *et al.* (1945) with *Fusaria*.

Factors influencing thiamine synthesis by molds have received scant attention. *Fusarium lini* Bolley was observed to contain 20 μg. while *F. graminearum* had a potency of only 5 μg. thiamine/g. (Vinson *et al.*, 1945) indicating species differences. Apparently cultural practices also affect its production. With *F. lini* Bolley, cocarboxylase was produced in greater amounts with potassium nitrate than with ammonium sulfate as the source of nitrogen (Wirth and Nord, 1942).

Media low or absent in chlorides and maintained between 24 and 25°C. were reported to favor thiamine elaboration by *Aspergillus oryzae*. Supplements of nucleic acid and sodium urate were more satisfactory than ammonium nitrate (Wirth and Nord, 1942).

3. *Synthesis from Precursors*

That microorganisms are capable of utilizing portions of the molecule to synthesize thiamine was indicated in the investigations of Pavcek *et al.* (1937) wherein it was observed that thiamine which had been destroyed by heat was regenerated by bakers' yeast. In the same year Knight (1937) reported that pyrimidine and thiazole could replace thiamine in the nutrition of *Staphylococcus aureus* and in 1938 Schultz *et al.* announced the biological activity of these compounds for yeasts requiring thiamine. Following these demonstrations numerous studies which have been reviewed extensively elsewhere (Knight, 1945; Schopfer, 1943) were undertaken with various fungi, protozoa, and higher plants.[1]

As a result of their investigations with yeasts, Schultz *et al.* (1941) developed a commercial method for increasing the thiamine content of bakers' yeast. In this process the pyrimidine and thiazole moieties were supplied to yeast mashes during propagation to provide cells varying from 300 to 1200 μg. thiamine/g. While bakers' strains couple these intermediates more efficiently than do other yeasts, Van Lanen *et al.* (1942) and Fink and Just (1942b) found that *Saccharomyces carlsbergensis*, *Saccharomyces ellipsoidus*, *Endomyces vernalis*, *Zygosaccharomyces pini*, *Torula utilis*, *Oidium lactis*, and *Aspergillus oryzae* also are active. With bakers' yeast utilization of pyrimidine and thiazole proceeds almost quantitatively until the cells attain a vitamin potency of 700 to 800 μg./g. above which the efficiency of conversion decreases sharply. When supplied with comparable amounts of intermediates the other yeasts ranged in efficiency from 6 to 65% (Van Lanen *et al.*, 1942).

The coupling of intermediates is markedly enhanced by aeration, a low temperature of incubation, and by a factor(s) contained in spent

[1] Cf. the review of Bonner and Bonner in this volume.

wort (Van Lanen *et al.*, 1942). Whether the factor(s) in wort is similar to or identical with that found by Kidder and Dewey (1942) to be essential for the coupling of pyrimidine and thiazole by protozoa is not known.

When provided with one intermediate in the absence of the other, yeasts vary in their ability to complete the synthesis. Schultz *et al.* (1941) found Fleischmann yeast strain 189 to be capable of synthesizing some thiamine from pyrimidine, while Van Lanen *et al.* (1942) Odintsova (1943) and Fink and Just (1942b and c) reported the formation of thiamine from thiazole but found negligible synthesis with pyrimidine alone.

In general, commercial yeasts are more exacting than most other fungi, e.g., *Phycomyces* species (Knight, 1945; Schopfer, 1943), with regard to the intermediates which they can utilize for growth or convert quantitatively to thiamine. No thiazoles other than 4-methyl, 5-β-hydroxyethylthiazole and the same in which the β-hydroxyl group has been replaced by an acyl group (Mead and Lee, 1943) have been found satisfactory. The patent of Harrison (1944), however, covers the use of thiazoles containing in the β-hydroxyl position either hydrolyzable or otherwise replaceable substituents. Yeast, but not *Staphylococcus aureus*, also is capable of converting *l*-α-amino-4-methyl-5-thiazolepropionic acid into 4-methyl-5-β-hydroxyethylthiazole (Harington and Moggridge, 1940).

The requirement for the pyrimidine moiety appears to be equally specific, with only certain substitutions in the 5 position permissible. Of these aminoethyl or hydroxymethyl (Ashida, 1942; Fink *et al.*, 1942), cyano (Harrison, 1944), aminomethyl (Fink and Just, 1942c), and thioformyl or ethoxymethyl (Schultz *et al.*, 1941) groups can be converted by various yeasts to thiamine through methylenic linkage with the nitrogen of thiazole.

4. Absorption by Microorganisms

That thiamine from the medium can be concentrated within the cells of microorganisms was first detected by Eijkman *et al.* (1922) who noted that both bakers' and brewers' yeasts removed the antineuritic principle from solution. Scheunert and Shieblich (1935) reached the same conclusion after noting that beer contained only small amounts of vitamin B whereas, when fortified after fermentation had been completed and the yeast separated no subsequent destruction occurred. Pavcek *et al.* (1936) and Fischer (1938) confirmed the behavior of yeasts with respect to thiamine and Schopfer (1943) extended the observation to *Phycomyces.* From these reports and their subsequent elaboration industrially feasible methods evolved for concentrating thiamine from natural products and

simultaneously preparing enriched yeasts. In one of these processes (Gorcica and Levine, 1942) thiamine was first extracted from such materials as wheat germ, rice polishings, or malt sprouts after which sugar was added to yield the desired amount of yeast of predetermined thiamine potency and the medium was fermented. In another method, Schultz *et al.* (1942) similarly proposed the enrichment of yeast employing crystalline thiamine. Yeasts can be enriched in this manner to give potencies of 650 μg. thiamine/g. with high efficiency (Fink and Just, 1941b; Schultz *et al.*, 1942) and cells have been fortified as high as 6260 μg./g. with lower efficiencies (Van Lanen *et al.*, 1942).

The abstraction of thiamine from solution by yeast takes place both aerobically and anaerobically in the presence of fermentable substrate but poorly under anerobic conditions in the absence of substrate. Fink and Just (1941b) noted that *Torula* yeast exhibits maximum absorption when introduced into 20% glucose concentrations which restrict fermentation. High temperatures, galactose or maltose (Fink and Just, 1941b), and alcohol (Sperber and Renvall, 1941) also enhance uptake while xylose and lactose are without effect. When the permeability of the cells is disturbed, thiamine uptake is decreased (Fink and Just, 1941b). Actually, in the presence of small amounts of salts, especially multivalent ions, absorbed thiamine may be excreted (Sperber, 1942).

Based on a similarity of the action of various inhibitors and on the optimum pH for absorption (3.5–4.0), Sperber (1942) has advanced the theory that yeast phosphatase is concerned with thiamine uptake. Iodacetate, azide, and fluoride completely inhibit absorption while cyanide is partially inhibitory. Both absorption and phosphatase activity likewise are retarded by 2-methyl, 4-amino, 5-methylaminopyrimidine.

Thiamine in the cells of microorganisms may be present in the free state, as the mono- or diphosphates, or as thiamine disulfide, the proportion of each form varying with the history of the cells. Hochberg *et al.* (1945) found that phosphorylated thiamine constituted from 17 to 93% of the thiamine found in commercial yeasts, the lower values being associated with thiamine enriched samples. Fink and Just, with enriched cells of *Torula utilis*, report figures up to 97% for the phosphorylated form (Fink and Just, 1942a). Upon drying (Hochberg *et al.*, 1945) or aging (Teysseire, 1945) yeast phosphatases are liberated which in turn decrease the amount of phosphorylated thiamine in the cells.

Myrbäck and Vallin (1944), from an examination of the thiamine and cocarboxylase potencies of various yeasts, have concluded that in cells propagated anaerobically, such as brewers' yeast, cocarboxylase predominates. In aerobic yeast, on the other hand, thiamine is largely

present in the form of the disulfide which results from cleavage of the thiazole ring and oxidation of the sulfur. Thiamine disulfide does not respond to the usual assay procedures. They suggest the following transition:

$$\text{Thiamine Esters} \underset{\text{Anaerobic Conditions}}{\overset{\text{Aerobic Conditions}}{\rightleftharpoons}} \text{Thiamine} \rightleftharpoons \text{Thiamine Disulfide}$$

Since thiamine esters are absent in strongly aerated cells and fermentative activity is correspondingly suppressed the above has been suggested as an explanation of the Pasteur effect. However, considerable evidence to support the conclusion that thiamine disulfide exists in insignificant amounts in yeast cells has been offered by Schonberg and Sperber (1945).

5. Thiamine Inactivation

Upon continued incubation especially under aerobic conditions, microorganisms destroy thiamine both in the cells and in the medium (Fink and Just, 1941a, 1942a; Kavanagh, 1942; Lewis, 1944; Van Lanen *et al.*, 1942). The enzymatic degradation of thiazole (Bonner and Buchman, 1939) and thiamine pyrophosphate (Westenbrink and Veldman, 1942) in plant tissues and yeast cells, respectively, already have been described. Thiamine is inactivated also by aquatic animals through cleavage of the methylene bridge (Krampitz and Woolley, 1944). Whether inactivation of thiamine by microbial cells is attributable to (a) enzymes similar to those mentioned above, (b) metabolic degradation such as has been noted in the case of the codehydrogenases, or (c) conversion to disulfide or other inactive forms is a subject for speculation. Nevertheless it is readily apparent that losses do occur and are one of the factors determining the thiamine potencies of various microorganisms.

III. Riboflavin

Riboflavin synthesis has been demonstrated qualitatively in a wide variety of microorganisms through growth studies. These have been adequately reviewed elsewhere (Knight, 1945; Peterson and Peterson, 1945). Table II summarizes typical results of quantitative studies of microbial biosynthesis. Of the bacteria none has been reported which exceeds the capacity of *Clostridium acetobutylicum* to produce this vitamin. Among the molds, few have been examined, but *Aspergillus niger* has been reported to excrete relatively large amounts of the vitamin. While commercial yeasts have long been considered rich sources other yeasts such as *Candida guilliermondia, C. flareri*, and the Ascomycetes,

Eremothecium ashbya and *Ashbya gossypii*, are capable of producing prodigious amounts.

In spite of the attention which has already been directed toward a fuller understanding of the factors affecting biosynthesis of riboflavin, only limited progress has been made. Some of the factors influencing synthesis by several important microorganisms are discussed below.

TABLE II

Riboflavin Content of Microorganisms and Whole Cultures

	Dry cells, μg./g.	Medium, μg./ml.	References
Bacteria			
Aerobacter aerogenes	....	3.7	236
Acetobacter suboxydans	....	8.5	319
Azotobacter vinelandii	305–350	......	149
Bacillus pasteurianum	15	......	256
Clostridium acetobutylicum	....	50[1]	183
Clostridium acidi-urici	14.3	......	18
Clostridium butyricum	130	......	256
Lactobacillus delbruckii	115	......	256
Mycobacterium smegmatis	8600	57.5	179
Mycobacterium tuberculosis	150–200	......	28, 33
Phytomonas tumefaciens	....	2.9	180
Yeasts			
Ashbya gossypii	....	1000	302
Candida arborea	46.5–69.5	......	3, 272
Candida flareri	....	216	303, 304
Candida guilliermondia	....	157	303, 304
Eremothecium ashbyii	....	1800	218a
Hansenula suaveolens	54	......	132
Mycotorula lipolytica	59	......	132
Oidium lactis	39.9–55.0	......	3, 272
Saccharomyces cerevisiae, (primary)	39.1–75	......	3, 199, 272
Saccharomyces cerevisiae (brewers')	36–42	......	199
Torula utilis	26–62	......	3, 132, 157, 272
Molds			
Aspergillus niger	....	5	147
Penicillium chrysogenum	39.8–47.5	......	301

1. *Synthesis by Bacteria*

Of all the B vitamins, microbial synthesis of riboflavin has been encountered most frequently. Members of the following genera have been reported to produce from 0.02 to 0.36 μg./ml. when cultured in riboflavin-free media: *Escherichia, Aerobacter, Klebsiella, Serratia, Proteus, Pseudomonas, Alkaligenes, Achromobacter, Chromobacterium,*

Flavobacterium, *Propionibacterium*, *Micrococcus*, *Staphylococcus* and *Bacillus* (Tittsler and Whittier, 1941). *Clostridium acidi-urici* (Barker and Peterson, 1944) grown in synthetic medium with uric acid as sole source of nitrogen produced 14.3 μg./g. dry cells. When cultured aerobically in distillers' thin stillage, *Aerobacter aerogenes* and *A. cloacae* multiplied the riboflavin content fivefold and threefold, respectively (Novak *et al.*, 1943). A fluorescent pigment formed by *Corynebacterium* also has been identified as riboflavin (Crowe, 1939).

Clostridium acetobutylicum. Riboflavin synthesis by this organism in the course of the butanol fermentation has been of industrial importance for several years and serves as an excellent example of the development of valuable by-products from fermentation residues. Recovery of wastes from this fermentation followed the observation by Miner (1940) that mashes fermented with *Cl. acetobutylicum* contained much more riboflavin than the original mash components, assaying from 25 to 62.5 μg./g. on a dry basis.

In grain fermentations the formation of an intense yellow pigment had been noted in 1927 by Weyer and Rettger, however, they attributed it to the solubilization of zein by the neutral solvents formed. Undoubtedly, these workers had observed the biosynthesis of this vitamin before it had been isolated or identified. At about the same time as Miner, others identified the yellow pigment which appeared in butanol fermentations of rice with the then newly-characterized vitamin (Yamasaki, 1939, 1943; Yamasaki and Yoshitome, 1938).

Following the demonstration that animal feeds required supplemental riboflavin, efforts were made to improve biosynthesis in commercial butanol fermentations. From various studies which have been made using different techniques it has been established that optimum riboflavin formation occurs only when the iron concentration of the medium is maintained between 1 and 3 p.p.m. This level has been determined by (a) determination of iron in various mashes, (b) the use of agents to render iron biologically unavailable, and (c) the addition of iron to deficient media. Whereas iron is very critical to riboflavin formation, only high concentrations adversely affect solvents production. By limiting the iron content of corn mashes, Arzberger (1943) obtained more than 3000 μg. riboflavin/g. dry residue. He found (Table III) that cobalt as well as iron inhibited appreciably while salts of zinc, copper, and lead were much less active.

In commercial butanol fermentations for the production of riboflavin concentrates, significant contamination of mashes with iron arises from the grain, steam and water supply, and from stillage when the latter is added to supply additional nutrients. Walton (1945) demonstrated

TABLE III

Effect of Metals on Riboflavin Synthesis by Clostridium acetobutylicum[1]

Compound added	Concentration, mg./l.	Solvent yield, % of dry corn	Riboflavin based on	
			Original corn, μg./g.	Dried filtrate, μg./g.
None	....	26.4	508	2720–3150
Ferrous sulfate	3.2	26.6	163	810
	32.0	27.8	39	197
	320.5	28.0	15	69
	3205.0	24.3	31	145
	.32050.0	3.2	38	33
Cobaltous acetate	3.2	27.7	105	620
	32.0	27.9	75	242
	320.5	12.8	32	64
Cupric acetate	3.2	31.0	...	3180
	32.0	31.0	387	2032
	320.5	6.7	25	41
Zinc acetate	3.2	27.6	603	3430
	32.0	29.4	539	2945
	320.5	29.2	462	2440
	3205.0	4.0	34	55
Lead acetate	3.2	29.6	460	2500
	32.0	29.8	407	2295
	320.5	28.4	422	2110
	3205.0	5.3	26	39

Mash composed of 5% cleaned, dry corn.
[1] From: Arzberger, C. F., U. S. Patent 2,326,425, 1943.

that rice which is relatively low in iron was an excellent supplement to corn and led to high yields of riboflavin.

Yamasaki (1941, 1942) also recognized the significance of iron in butanol fermentations and observed that the elaboration of riboflavin was increased by adding from 0.1 to 2% calcium carbonate or calcium acetate during the first 7 hours of fermentation. This effect is probably due to the absorption of iron by these salts since a similar principle was employed by Steinberg (1935, 1938, 1939) to prepare media deficient in trace elements. By adding from 0.005 to 0.05% sodium sulfite to grain mashes, Legg and Beesch (1945) substantially increased riboflavin synthesis.

In a study of various aerobic and anaerobic bacteria, Saunders and McClung (1943) noted that addition of traces of iron as ferrous sulfate stimulated riboflavin production in the case of four of five strains of *Cl. acetobutylicum*. These results should not be considered at variance

with those above since the iron content of grain and water differ with the locality. Also, slightly different optimal iron levels probably exist for different strains of the species. Saunders and McClung, confirming Rodgers (1942), noted that the stimulatory effect of iron appeared to be limited to strains of *Cl. acetobutylicum* because *Cl. roseum*, *Cl. felsineum* and other pigmented anaerobes, including certain yellow butyric types, did not respond to the iron supplements although these latter anaerobes do not produce large amounts of riboflavin.

Some strains of *Cl. acetobutylicum* actively ferment lactose with the elaboration of substantial amounts of riboflavin. Whey or skim milk, however, do not support vigorous or complete fermentation unless iron is added. Meade *et al.* (1945) found that whey fermented after adjustment of the iron content to 0.5 to 4.5 p.p.m. gave yields of riboflavin of 50 or more μg./ml. Within these limits of iron concentration the actual optimum for riboflavin synthesis was found to vary with the pH, and the acids used (Pollard *et al.*, 1947; Rogers *et al.*, 1947). With iron controlled, the addition of 0.5 to 2.0% xylose to whey was observed to increase further the synthesis of this vitamin (Meade *et al.*, 1947). As with grain mashes, iron is much more critical to riboflavin synthesis than to solvents production.

Hickey (1945, 1947) has investigated the ability of organic compounds, frequently used in analytical procedures, to render iron biologically unavailable to *Cl. acetobutylicum*. Of these cupferron, 7-iodo, 8-hydroxyqüinoline, 5-sulfonic acid, and the anions, cyanide, oxalate, thiocyanate, sulfide, and citrate were ineffective. However, 2,2′-dipyridine was employed successfully in corn mashes to which iron had been added. Hickey observed that 9.5 μg. of dipyridine were required to bind 1 μg. of ferrous iron whereas stoichiometrically, 8.4 μg. are needed. The slight excess was thought necessary to suppress dissociation of the iron complex. Dipyridine did not increase riboflavin production by two molasses fermenting strains of *Cl. acetobutylicum*. This was attributed to peculiarities of these strains or to inhibition by substances other than iron. On the other hand, iron may occur in a form not readily reactive with dipyridine, although biologically available to the organism.

An explanation of the action of iron in *Cl. acetobutylicum* fermentations has been proposed by Leviton (1946). He noted that riboflavin, although stable in pure solutions to hydrogen peroxide, is readily decomposed by dilute solutions of this reagent when traces of ferrous iron are present. Between iron levels of 0.018 and 0.054* mg. atoms/l. (about 1 to 3 p.p.m. of iron) the rate constants for riboflavin destruction rose from 2.3 to

* Leviton (personal communication) corrected to 0.1 those iron concentrations given originally (1946).

18.9%/hour. It will be recalled that the higher level of iron is approximately that which is critical to riboflavin accumulation in cultures of the butyl anaerobes. Leviton found further that catalase, potassium iodide, and sulfite which accelerate the decomposition of H_2O_2 stabilized riboflavin. This evidence along with the known functions of enzymes in which flavin-adenine-phosphoric acid comprise the prosthetic group has been put forth to support the conclusion that H_2O_2 is produced by *Cl. acetobutylicum* although its formation has not been demonstrated. Whether the action of iron can be explained (a) by its capacity to destroy riboflavin when in combination with H_2O_2 as described above, or (b) by permitting an alternative and preferred respiratory mechanism to function (Hickey, 1945), the involvement of iron porphyrin catalysts is indicated. This suggests the need to reinvestigate the generally accepted belief that all obligate anaerobes are devoid of these systems.

Considerable quantities of riboflavin likewise are produced by *Mycobacterium smegmatis* (Mayer and Rodbart, 1946). Unlike *Cl. acetobutylicum* synthesis is not affected by iron. In Long's medium, yields of more than 20 μg./ml. were formed in 8–12 days. When glycine replaced asparagin in the above medium, both growth and vitamin synthesis were depressed. Other amino acids were without effect, but with nitrate or ammonium salts as the only nitrogen source potencies up to 57 μg./ml., equivalent to 8600 μg./g. dry cells, were secured. Glycerol in the original medium was replaceable by levulose but not glucose although the latter supported good growth.

Mycobacterium tuberculosis produced riboflavin but in somewhat lesser amounts (Boissevain *et al.*, 1938; Rohner and Roulet, 1939), cultures assaying 2.9 μg./ml. having been obtained.

2. *Synthesis by Yeasts*

The general yeasts of commerce such as those for food and baking purposes contain from 40 to 85 μg. riboflavin/g. The quantity withheld by the cells during propagation is apparently rather constant whereas variable amounts are released into the medium. Massock (1943) and Van Lanen (unpublished) were unable to alter appreciably either riboflavin synthesis or retention by *Saccharomyces cerevisiae* by varying culture conditions. However, according to Pett (1935) the content of *S. cerevisiae* can be raised by culturing in the presence of compounds such as KCN, cysteine, and pyridine which affect the activity of the iron-containing catalysts. It was observed further by Pett that aerobic conditions lower the riboflavin content of yeast cells. This has been substantiated by Singh *et al.* (1947) who found that cells produced without aeration or agitation (low cell crop) contained 108 μg. while those aerated (high cell crop) contained 60–65 μg. riboflavin/g.

Iron also influences riboflavin synthesis by *Torula utilis* but less markedly than with some of the organisms discussed above. Lewis (1944) found that cells propagated in synthetic medium in the absence of iron were slightly higher in content than when iron was supplied. Of interest in connection with this organism is the fact that *T. utilis* was higher in riboflavin content from synthetic medium than from fruit juice (Lewis *et al.*, 1944) or other media (National Research Council, 1943).

In 1943 and subsequently Burkholder described an interesting species, *Candida guilliermondia*, which is capable of outstanding synthesis of riboflavin (1943a and b, 1944). In a medium composed of 2% glucose, 0.2% ammonium sulfate and other inorganic salts yields up to 40 μg./ml. were produced, and an increase of approximately 50% resulted when certain amino acids were added. Other investigators found this organism to vary considerably in riboflavin-producing ability, the explanation for which was disclosed by Tanner *et al.* (1945, 1947b) who established that iron again was the major controlling factor in this fermentation. The optimum iron level for riboflavin synthesis, however, was found to be in the neighborhood of 0.005 to 0.01 p.p.m. compared with 1–3 p.p.m. for *Clostridium acetobutylicum*. When precautions were taken to regulate the iron concentration, yields up to 216 μg. riboflavin/ml. were secured with *Candida* species. Waring and Werkman (1943) have shown that in cells of *Aerobacter indologenes* produced on nutrient solutions depleted of iron, catalase activity was greatly reduced and cytochrome could not be demonstrated.

Among the *Ascomycetes* are two yeastlike organisms, *Eremothecium ashbyii* and *Ashbya gossypii*, which are capable of synthesizing extraordinary amounts of riboflavin. These organisms, both plant pathogens, have been described by Guilliermond (1928, 1936). They have many characteristics in common including the formation of short, fine-structured mycelia but can be differentiated morphologically principally by the shape of their ascospores (Guilliermond, 1936). In the course of studying *E. ashbyii*, Guilliermond *et al.* (1935) noted that a greenish-yellow pigment was formed on Gorodkowa or Sabouraud agar and crystals of the pigment frequently were observed in cell vacuoles. Physical and chemical properties indicated a similarity to riboflavin and confirmation of its identity with this vitamin was obtained by Raffy (1937) and Miramanoff and Raffy (1938a and c).

The French workers obtained appreciable synthesis of riboflavin with *Eremothecium ashbyii* (112 μg./g. agar medium and 159 μg./ml. liquid medium) but incubation periods as long as 24 days were necessary (Raffy and Fontaine, 1939; Renaud and Lachaux, 1944, 1945). The requirement for aerobic conditions, likewise, was recognized (Raffy,

1939), a feature which has been extensively elaborated upon by later investigators (Foster, 1947; Piersma, 1945; Rudert, 1945; Stiles).

Schopfer (1944) called attention to biochemical differences separating *Eremothecium ashbyii* from *Ashbya gossypii* and confirmed Guilliermond *et al.* (1935) with regard to the fact that only trace amounts of riboflavin are formed by the latter organism. A further contrast has been drawn on the basis of growth requirements since *E. ashybyii* requires accessory factors over and above thiamine, biotin, and inositol which are needed by both organisms (K gl and Fries, 1937; Robbins and Schmidt, 1939). The accessory substance for *E. ashbyii* is contained in norite-treated peptone and is partially replaceable by leucine and arginine (Schopfer and Guilloud, 1945a).

Despite the definitely-established need for aerobic conditions for maximum riboflavin elaboration by *Eremothecium ashbyii*, submerged, aerobic techniques were not applied until visualized in 1941 by Mayer (1946). By this method yields of 80 μg./ml. were secured and the commercial possibilities of this organism were indicated. Several patents followed employing processes basically related in their adaptation to submerged conditions, but utilizing as the fermentable substrate various proteinaceous substances with carbohydrates (Foster, 1947; Piersma, 1945; Stiles) or lipides (Rudert, 1945). Yields as high as 400 μg./ml. in 160 hours were reported. Moore and de Becze (1947), by fermenting supplemented ethanol stillage with *E. ashbyii*, prepared residues containing 4000 μg. of riboflavin/g. on a dry basis.

In contrast to earlier reports (Guilliermond, 1935; Schopfer and Guilloud, 1945b, *Ashbya gossypii* has been shown to be capable under certain conditions of riboflavin synthesis of the same order of magnitude as *Eremothecium ashbyii*. Wickerham *et al.* (1946) obtained potencies up to 381 μg./ml. in a medium of glucose, peptone, and yeast extract. Tanner and Van Lanen (1947a) discussed the factors influencing vitamin production by this organism and reported yields of 1000 μg./ml. in a medium composed of 0.5% corn steep liquor solids, 0.5% animal tankage or peptone, and 2–4% glucose.

Although neither *Eremothecium ashbyii* nor *Ashbya gossypii* is affected by iron in the concentrations normally encountered, elaboration of the vitamin by the latter organism is markedly inhibited by certain substances, probably organic in nature, which occur in various natural products.

3. *Synthesis by Molds*

The formation by *Aspergillus niger* in Raulin's solution of a water-soluble yellow pigment with fluorescent properties and subsequently

characterized as riboflavin has been described in a series of papers by Lavollay and Laborey (1937, 1941). Maximum yields, in the order of 5 μg./ml. resulted when the medium contained no more than 0.4 mg. % of a magnesium salt. Ascorbic acid suppressed synthesis almost completely. Knobloch and Sellmann (1941) reported that numerous strains of *A. niger* elaborate a flavin-type pigment, notably when nitrogen is supplied as magnesium nitrate.

Peltier and Borchers (1947) have made a study of the ability of molds to synthesize riboflavin when cultured on wheat bran. All of more than two hundred isolates tested produced some riboflavin with certain *Aspergilli* and *Fusaria* giving the highest yields. One strain of *Aspergillus* increased the bran from an initial value of about 3 μg./g. to 58 μg./g.

IV. Pantothenic Acid

The term pantothenic acid was coined to denote the universal distribution of a substance recognized in 1933 to have remarkable growth-promoting action for certain strains of *Saccharomyces cerevisiae* (Williams *et al.*, 1933). Even at this early date, synthesis by *Aspergillus niger* and *Bacillus subtilis* had been observed.

The function of pantothenic acid has only recently been elucidated with the demonstration that it is a component of coenzyme A. This coenzyme functions in the second or acetate phase of pyruvate oxidation in which acetyl phosphate is formed as well as in other acetylation reactions (Lipmann and Tuttle, 1945). Coenzyme A contains approximately 11% pantothenic acid or 0.7 μg. per enzyme unit (Lipmann *et al.*, 1947a, 1947b). That pantothenic acid might be concerned with pyruvate oxidation was suggested by Dorfman *et al.* in 1942 and confirmed by Hills (1943).

Coenzyme A has been found in all microorganisms examined including species of *Lactobacillus*, *Propionibacterium*, *Escherichia*, *Proteus* and *Clostridium* (Novelli and Lipmann, 1947a). With *L. arabinosus* pantothenic acid in the form of coenzyme accounted for 90% of the total in the cells. Digestion with clarase and papain, a conventional procedure for liberating pantothenic acid, hydrolyzes the coenzyme slowly. Better success has been reported using a phosphodiesterase combined with an unidentified enzyme from liver (Lipmann and Kaplan, 1946; Lipmann *et al.*, 1947a). The requirement for both enzymes suggests that at least two linkages bind pantothenic acid in the coenzyme.

1. *Synthesis by Microorganisms*

Table IV summarizes the pantothenic acid contents of those organisms which have been studied quantitatively. It may be seen that

bacteria vary appreciably both with regard to the amounts produced and the degree of excretion, with *Aerobacter aerogenes* cultured anaerobically being the richest.

TABLE IV

Pantothenic Acid Content of Cells and Whole Cultures

	Dry Cells, μg./g.	Total synthesized, μg./g. dry cells	References
Bacteria			
Aerobacter aerogenes (aerobic)	140	780	315
Aerobacter aerogenes (anaerobic)	340	750	315
Azotobacter vinelandii	152–184	....	149
Clostridium butylicum	93	318	315
Phytomonas tumefaciens	41	....	180
Proteus vulgaris	100	100–130	315
Pseudomonas fluorescens	91	311	315
Serratia marcescens	120	172	315
Yeasts			
Saccharomyces cerevisiae (aerobic)	118	....	176
Saccharomyces cerevisiae (anaerobic)	198	....	176
Hansenula suaveolens	180	....	132
Torula utilis	86–180	....	132, 157, 199
Commercial foil yeast	120–180	....	176
Commercial bakers' yeast	180–330	....	176
Commercial brewers' yeast	100	....	199
Debittered brewers' yeast	72–86	....	199
Fungi			
Penicillium chrysogenum	107–140	....	301
Penicillium chrysogenum	201–212	....	200

Results of analysis of commercial yeasts show that foil yeast is significantly lower in pantothenic acid than bakers' types. The latter are differentiated from foil yeast by having a higher nitrogen content and higher activity. Anaerobically-propagated yeasts were found to have nearly double the level of aerobic cells (Massock and Baldwin, 1943) which is similar to experience with *A. aerogenes* (Thompson, 1942) That this does not hold for brewers' yeasts which are low in pantothenic acid is probably attributable to the fact that drying and debittering processes effect appreciable destruction.

Penicillium chrysogenum, one of the few molds studied synthesizes relatively large quantities of pantothenic acid. During fermentation of corn steep liquor-lactose medium this organism increased the level in the medium from 1.8 to nearly 18 μg./ml. (Tanner *et al.*, 1945). That

found only in the mycelium ranges from 108 to 200 μg./g. (Newell *et al.*, 1947; Tanner *et al.*, 1945).

Whereas synthesized pantothenic acid is generally excreted to a considerable extent, absorption is accomplished and the absorbed vitamin is converted to coenzyme A by species which require an external source (Novelli and Lipmann, 1947b).

2. *Synthesis from Precursors*

Prior to the full elucidation of the structure of pantothenic acid, the ability of β-alanine to replace it in the growth of certain organisms had been observed. Subsequently, it was established that numerous organisms can accomplish the synthesis of pantothenic acid when provided with either β-alanine (Mueller, 1933; Mueller and Klotz, 1938) or pantoyl lactone (Ryan *et al.*, 1945; Underkofler *et al.*, 1943; Woolley, 1939). The promotion of pantothenic acid formation by *Saccharomyces cerevisiae* by supplementing the medium with β-alanine was the subject of a patent by Schultz *et al.* (1945). Most of the lactic acid bacteria are more exacting, however, and thus are unable to utilize these compounds (Cheldelin *et al.*, 1945; Snell *et al.*, 1938).

A cell-free system prepared from yeast which is capable of coupling β-alanine and pantoyl lactone has been described by Wieland and Moller (1941). Ammonium ions activated the system, 0.1 *M* producing a maximum response (Wieland and Moller, 1942).

Kuhn and Wieland (1942) have speculated that biosynthesis of pantothenic acid might stem from valine and aspartic acid as follows: Valine by oxidative deamination is convertible to dimethylpyruvic acid which may than be condensed with formaldehyde to α-keto-β, β-dimethyl-γ-hydroxybutyric acid. The latter readily undergoes lactonization and upon reduction gives α, γ-dihydroxy-β, β-dimethylbutyric acid which yeast can combine with β-alanine to give pantothenic acid. Aspartic acid could give rise to β-alanine through decarboxylation (Kuhn and Wieland, 1942; Ravel and Shive, 1946).

V. Niacin

1. *Content of Microorganisms*

Compared with the tissues of higher plants and animals, microorganisms are not only richer in niacin but, despite fundamental differences in metabolism, they show remarkable uniformity. This may be taken as a reflection of the importance of this vitamin and corresponding coenzymes to their general economy. Analogous with thiamine, the

synthesis, absorption, excretion, and degradation of niacin have been shown to be operative in microbial cells and, as elaborated upon below, each functions in determining cell potencies. In view of the dynamic state which exists within the cell with respect to this vitamin, the degree of uniformity in potency is all the more noteworthy.

In Table V, values are given for the niacin content of various bacteria, yeasts, and molds. It is evident that, while marked by constancy in content, there are variations both within and between species. From

TABLE V

Niacin Content of Microorganisms and Culture Liquors

	Dry cells, μg./g.	Total synthesized in cells and medium, μg./g. dry cells	References
Bacteria			
Aerobacter aerogenes (anaerobic)	200	630	315
Aerobacter aerogenes (aerobic)	240	258	315
Azotobacter chroococcum	590	....	149
Clostridium butylicum	250	1930	315
Proteus vulgaris	250	330	315
Pseudomonas fluorescens	210	560	315
Serratia marcescens	240	470	315
Yeasts			
Candida arborea	157–580	....	3, 272
Candida guilliermondia	515	....	320
Hansenula suaveolens	590	....	132
Monilia murrmanica	272–375	....	223
Mycotorula lipolytica	600	....	132
Oidium lactis	195–248	....	3, 272
Saccharomyces cerevisiae (anaerobic)	190	....	320
Saccharomyces cerevisiae (aerobic)	585	....	320
Saccharomyces cerevisiae (enriched)	2930	....	176
Torula lactosa	447	....	320
Torula utilis	210–535	....	39, 70, 132, 300, 314, 320
Commercial foil yeast	180–400	....	177
Commercial bakers' yeast	200–700	....	39, 70, 132, 177, 199, 266
Commercial brewers' yeast	320–1000	....	39, 172, 266, 300, 327
Molds			
Penicillium chrysogenum	150–212	....	200, 301
Penicillium notatum	180	....	200

the evidence available to date, these differences can be ascribed to environmental factors and to strain characteristics which affect both synthesis and the retentive properties of organisms for this vitamin. Except for *Azotobacter chroococcum,* the bacteria and molds which have been studied are lower in potency than the majority of the yeasts. Certain bacteria synthesize appreciable amounts of niacin but, as shown later, it is largely excreted into the medium. Yeasts propagated anaerobically become enriched in niacin when this vitamin is present in the medium but are of low potency when it is omitted. (Compare brewers' yeast with *Saccharomyces cerevisiae* grown aerobically and anaerobically in synthetic medium.) These and similar data substantiate the view that synthesis is limited while absorption is unaffected or improved under anaerobic conditions. Certain organisms such as *Candida arborea* have been found to vary in niacin potency depending upon the substrate whereas *Oidium lactis* is uniformly low. Explanation for these differences may reside in the limited ability of the former species to synthesize and of the latter to accomplish either synthesis or absorption. Several types of yeasts grown in molasses-malt extract medium have been shown (Van Lanen, 1946) to vary in niacin content with *Candida guilliermondia* and a strain of distillers' yeast giving the highest potencies. While all lowered the level in the medium, presumably through absorption, with only the two above species was the final niacin in cells and medium equivalent or greater than that initially present. Despite synthesis which undoubtedly occurred, it is clear then, that destruction took place and in most instances exceeded the rate of synthesis.

2. Synthesis of Niacin

Complete synthesis of niacin has been demonstrated by microorganisms cultivated on inorganic carbon and salts (O'Kane, 1942), sucrose, atmospheric nitrogen and salts (Lee and Burris, 1943), acetic acid, inorganic nitrogen and salts (Fink and Just, 1939) as well as from various other synthetic preparations.

Although produced from simple carbon and nitrogen compounds, the pathway of niacin synthesis is still obscure. Numerous possible precursors have been examined with preponderantly negative results. It has been noted by Euler *et al.* (1944) that the requirement of *Proteus vulgaris* for niacin is satisfied by the tetrahydro (guvacin) and hexahydro derivatives, but obviously synthesis need not proceed via these compounds. From an interesting study by Bonner and Beadle (1946) it was concluded that since each of three genetically-distinguishable *Neurospora* mutants was capable of effecting a different step in the formation of niacin at least as many intermediates were involved. One of these was

isolated and found to have the empirical formula $C_8H_9HNO_3$ (niacin-$C_6H_5NO_2$). Recent studies (Beadle *et al.*, 1947) have shown that *Neurospora* synthesizes niacin from tryptophan via kynurenine and hydroxyanthranilic acid (Beadle *et al.*, 1947; Bonner, 1948; Mitchell and Nyc, 1948).

Of numerous organisms which have been surveyed, *Mycobacterium tuberculosis* appears to be the outstanding producer of niacin. Synthetic medium in which this organism was grown for 21 days was increased in content from 0.07 μg. to 37 μg./ml. in the cell-free liquor and to 61 μg./ml. in the whole culture (Bird, 1947). Cultures of *Bacillus vulgatus* and *Bacillus mesentericus* also accumulate considerable niacin according to Burkholder and McVeigh (1942). Per gram of moist cells, *Bacillus vulgatus* produced 709 μg. and *Bacillus mesentericus* 207 μg., however, the concentrations reached were much lower than with *Mycobacterium tuberculosis*. Less striking, but definite evidence of synthesis by other bacteria as well as by *Oidium lactis, Penicillium camemberti* and unidentified yeasts have been described by Burkholder *et al.* (1943, 1944). Examination of niacin production by obligate anaerobes has been confined to *Clostridium butylicum* (Thompson, 1942). This organism elaborates appreciable amounts which are largely excreted into the medium.

Environmental factors and medium components which alter the degree of niacin synthesis have been recorded by several investigators, the full significance and explanation of which must await further study. Bakers' yeast which has been propagated aerobically in the presence of yeast extract (Van Lanen, 1946) or malt extract (Massock, 1943) is reduced in niacin potency. This depressing effect is unexpected since both of these materials are favorable to growth. It may be due to substances which limit synthesis, reduce the metabolic requirement, or enhance destruction of niacin. Lewis (1944) has found that the production of niacin by *Torula utilis* is increased by cultivation in the absence of iron. This is similar to observations with riboflavin (discussed elsewhere). However, the analogy with riboflavin is not complete since Eppright and Williams (1946) showed that the presence of KCN which has a favorable effect upon riboflavin production caused a reduction in niacin synthesis.

Of the factors which increase niacin synthesis, the finding that asparagin is stimulatory in synthetic medium (Van Lanen, 1946) is of special interest in view of the disclosure by Bovarnick (1943) that niacin or a compound with similar properties is formed by autoclaving asparagin in the presence of amino acids. Thiamine, likewise, improves the formation of niacin by *Aerobacter aerogenes* (Thompson, 1942) while

camphor and sulfaguanidine increase either its synthesis or retention (Eppright and Williams, 1946) by yeasts.

The importance of aerobic conditions for the elaboration of niacin by both yeasts and molds has been well established. Thompson (1942) found that *Aerobacter aerogenes* grown anaerobically produced only 41 percent of that obtained aerobically. Likewise, with bakers' yeast cultured in synthetic medium, aerobic cells were not only more than threefold richer in niacin but they excreted appreciably more vitamin into the medium (Van Lanen, 1946). The results of Singh *et al.* (1947) with various yeasts also suggest a correlation between the efficiency of aeration and niacin production.

3. Absorption from the Medium

Niacin, like thiamine, is readily absorbed from solution by yeasts, and probably by other microorganisms as well. Although of both practical and theoretical interest, it has not been determined how efficiently absorbed niacin is converted to the usual nucleotides. Under optimum conditions as much as 90% of the medium niacin may be concentrated in the cells (Massock, 1943; Van Lanen, 1946) and cell potencies of about 3 mg. of niacin/g. have been obtained (Massock, 1943). Such factors as (a) the composition of the medium, (b) availability of the vitamin, (c) conditions of cultivating the yeast, and (d) the particular species employed have been shown to alter the efficiency of absorption.

Contrasted with thiamine, absorption of niacin by yeast is dependent upon the presence of a fermentable substrate and excretion readily occurs following the utilization of the available carbohydrate (Van Lanen, 1946). Some forms of naturally-occurring niacin are not acquired by yeast despite their availability to such assay organisms as *Lactobacillus arabinosus.* Failure to permeate the cells may be responsible since it will be recalled that cocarboxylase is unavailable to some yeasts perhaps for this reason. Of the various types of yeast investigated, commercial bakers' yeast, and a distillers' grain yeast absorbed niacin most efficiently (Van Lanen, 1946). Because synthesis is favored by aerobic conditions whereas absorption occurs best anaerobically, enrichment of yeasts would be preferably accomplished by propagation aerobically followed by exposure to anaerobic conditions in the presence of fermentable substrate and available forms of the vitamin.

4. Niacin Inactivation

Harden and Young in their initial studies on codehydrogenase called attention to the lability of this coenzyme when it was present in yeast juice (1906). Subsequent studies with various organisms and tissues

indicate that the degradation of coenzymes I and II is common during active metabolism. Morel working with *Proteus vulgaris* (1941) found that the metabolic rate of this organism could be maintained only by the addition of nicotinamide. She attributed the utilization and need for this compound to a "wearing out" process which was a function of the number of oxidations and reductions undergone by the coenzyme. The inactivation of coenzyme in animal tissues results apparently from the splitting-off of macinamide, but Handler and Klein (1942) were unable to retard the degradation by supplementing with this compound.

From an examination of the niacin content of yeasts during aerobic and anaerobic propagation as well as from the analysis of various fermentation residues it may be seen that generally a net loss of niacin occurs, although in most instances the organisms employed are capable of its synthesis. Comparison of a single strain under aerobic and anaerobic conditions revealed that destruction was accelerated anaerobically (Van Lanen, 1946). Similarly, Swaminathan (1942) has observed that distillers' yeast is much lower in this vitamin than *Torula* or pressed yeast which was propagated aerobically. Since cells and culture liquors were analyzed following the liberation of bound niacin, it must be concluded that the metabolic breakdown of codehydrogenase proceeds further involving destruction of niacin itself. This destruction is probably correlated with accelerated codehydrogenase activity as occurs during fermentation. This utilization is distinguishable, quantitatively at least, from that described by Koser and Baird (1944) and Allinson (1943) in which niacin supplied both carbon and nitrogen for certain organisms. Nevertheless, it is readily apparent that by degradation the niacin level of microbial cells may be appreciably altered.

VI. Biotin

Biotin was first isolated from egg yolk in 1935 by Kögl and the isolation procedure was described in detail by Kögl and Tonnis in 1936. Later, Melville *et al.* (1942) and du Vigneaud *et al.* (1941) accomplished the isolation from milk and liver, respectively. The structure of biotin from liver has been established by du Vigneaud and coworkers; however, another structure, the existence of which has been questioned (Melville, 1944), has been proposed for the biotin from egg yolk. Hofmann (1945) and Melville (1944) have reviewed the chemistry and physiology of biotin.

Evidence recently accumulated indicates that biotin is linked in function with certain carboxylation, decarboxylation, and deamination reactions. The first insight into its biochemical role came from the observation that biotin, when present in relatively high concentration,

eliminated the requirement of *Lactobacilli* and *Streptococcus fecalis* for aspartic acid (Stokes *et al.*, 1947a and b). *L. arabinosus*, in addition, is capable of utilizing oxalacetic acid in the absence of both biotin and aspartic acid, in which case biotin-deficient cells are evidently capable of converting oxalacetic acid to aspartic acid by transamination (Landy, 1947). Similarly with *Escherichia coli*, failure to synthesize biotin is reflected in a reduced formation of α-ketoglutaric acid, the latter being involved, also, in aspartic acid production (Shive and Rogers, 1947). With biotin depleted cells, Lichstein and Umbreit (1947a) have demonstrated further that the decarboxylation of aspartic, malic, and oxalacetic acids and the deamination of aspartic acid, threonine, and serine (1947b) are retarded. These functions can be accelerated by the addition of biotin.

1. Biotin in Microorganisms

Studies on the quantitative distribution of biotin in microorganisms have not been extensive; available data are summarized in Table VI.

TABLE VI

Biotin Content of Cells and Whole Cultures

	Dry cells, μg./g.	Total synthesized, μg./g. dry cells	References
Bacteria			
Aerobacter aerogenes (aerobic)	3.9	47.9	315
Aerobacter aerogenes (anaerobic)	2.4	13.4	315
Azotobacter vinelandii	2.56–4.23	....	149
Clostridium butylicum	1.7	1.8	315
Proteus vulgaris	3.4	24.1	315
Pseudomonas fluorescens	7.1	68.1	315
Serratia marcescens	4.1	34.1	315
Yeast			
Candida arborea	0.32	....	272
Mycotorula lipolytica	1.8	....	132
Saccharomyces cerevisiae	0.5–1.8	....	176, 272
Torula utilis	1.06–1.9	....	132, 157, 272
Fungi			
Penicillium chrysogenum	0.57–1.5	....	200, 301

Bacteria are particularly good sources of biotin with molds and yeasts being, in general, somewhat lower in potency. *Pseudomonas fluorescens* has been found to be one of the most productive with cells assaying 7.1 μg./g. (Thompson, 1942). *Torula utilis* cultured in fruit juices

(Lewis *et al.*, 1944), molasses (Singh *et al.*, 1947), and wood sugar stillage (Kurth and Cheldelin, 1946) ranged from 1.0 to 1.9 μg./g. *Saccharomyces cerevisiae* from molasses (Massock, 1943; Massock and Baldwin, 1943; Singh *et al.*, 1947) attained approximately the same potencies (0.5–1.5 μg./g.) while *Mycotorula lipolytica* and *Hansenula suaveolens* from wood sugars were slightly higher. On the other hand, *Candida arborea* produced significantly lower quantities than other yeasts (Singh *et al.*, 1947).

Among the molds, the mycelium of *Penicillium chrysogenum* grown on corn steep-lactose medium showed about the same level of biotin as the yeasts (0.57 to 1.5 μg./g.).

Various bacteria cultured on biotin-free media elaborate biotin (Landy and Dicken, 1941; McIntire *et al.*, 1941; Wright and Skeggs, 1944). Culture liquors from such organisms have been observed to range from 0.012 to 0.05 μg./ml., but substantially greater synthesis has been effected during the fermentation of sugar to fumaric acid by a *Rhizopus* species. In one instance, 1 g. biotin (equivalent to 0.13 μg./ml.) was recovered from 2000 gallons of fermented medium (Schivek, 1944).

Avidin, a protein found in raw egg white which forms a stable complex with biotin and certain biotin analogues (Eakin and Williams, 1940; du Vigneaud *et al.*, 1942b) inhibits the growth of those species which require an exogenous biotin supply, but has not been shown to enhance biotin production by those capable of its synthesis.

2. *Absorption by Microbial Cells*

During a survey of the vitamin content of commercial yeasts, Massock (1943) observed an appreciable variation in biotin content. The possibility of absorption from the medium was investigated as an explanation with the finding that as much as 90% of the biotin present in the medium could be removed by the cells. Consequently, cane molasses which contains from five to ten times as much biotin as beet molasses gives rise to cells of higher potency.

Bacteria which are heterotrophic for biotin absorb it in amounts which do not greatly exceed their metabolic requirements and inactivate that added in excess (Krueger and Peterson, 1948; Thompson, 1942). Of the total produced by bacteria capable of synthesis, amounts ranging from 10 to 98% have been found in the medium (Landy and Dicken 1941; McIntire *et al.*, 1941; Thompson, 1942). Therefore, compared with yeasts, bacteria have a lower excretion threshold with respect to this vitamin.

3. *Biotin Analogues*

Oxybiotin (*O*-heterobiotin), an analogue of biotin in which the sulfur is replaced by oxygen, has been synthesized (Duschinsky *et al.*, 1945; Hofmann, 1943, 1945) and its ability to promote the growth of various

organisms requiring biotin has been followed by several investigators (Duschinsky *et al.*, 1945; Krueger and Peterson 1948; Pilgrim *et al.*, 1945; Rubin *et al.*, 1945; Ryan *et al.*, 1945). These studies have revealed that *dl*-oxybiotin compared with *d*-biotin is 50% as active for *Lactobacillus arabinosus* and *L. pentosus*, 40% as active for *L. casei*, and only from 1–25 % as active for *Saccharomyces cerevisiae* and *Rhizobium trifolii*. Only *d*-biotin is biologically active (Stokes and Gunness, 1945, and if true for oxybiotin it has essentially equivalent activity for the two *Lactobacilli* named above but lower activity for the other organisms.

Whether the oxygen analogue is utilized per se or is converted to biotin has been a subject of considerable controversy (Hofmann and Winnick, 1945; Rubin *et al.*, 1945). Based upon differential assays following treatment of preparations to eliminate either biotin or oxybiotin, impressive evidence has been offered that oxybiotin is not converted to biotin by yeast (Axelrod *et al.*, 1946; 1947; Hofmann *et al.*, 1945, 1947) or by lactobacilli (Krueger and Peterson, 1948).

Desthiobiotin, which is obtained by cleaving the thiophene ring and replacing the sulfur with two hydrogen atoms, is the only biotin analogue possessing yeast growth activity comparable to biotin (Lilly and Leonian, 1944; Melville *et al.*, 1943). In contrast to oxybiotin, desthiobiotin when utilized is converted to biotin (Dittmer *et al.*, 1944; Leonian and Lilly, 1945). With *Escherichia coli* this reaction is prevented by 2-oxo-4-imidazolidinecaproic acid. Since an x-ray mutant of *Penicillium chrysogenum* synthesized desthiobiotin but was incapable of completing the synthesis to biotin, Tatum has suggested that the former may be a normal intermediate in biotin formation (1944, 1945). With the same organism, it was demonstrated that pimelic acid stimulated desthiobiotin production (Tatum, 1945) apparently giving rise to the valeric acid side-chain. Early reports that this compound stimulated biotin synthesis by diphtheria bacilli (du Vigneaud, 1942a) and *Aspergillus niger* (Eakin and Eakin, 1942) were thus confirmed. However, *Ashbya gossypii*, *Neurospora*, *Ceretostomella* and certain *Fusarium* species are unable to utilize pimelic acid to produce biotin (Robbins and Ma, 1942).

Seven 1,3-disubstituted ureas which may be looked upon as open chain analogues of desthiobiotin were biologically inactive for both *Lactobacillus casei* and *L. arabinosus* (Schultz, 1947).

VII. *p*-Aminobenzoic Acid

1. *Production by Microorganisms*

Recent reviews (Ansbacher, 1944; Sevag, 1946) have been devoted wholly or in part to considerations of the role of *p*-aminobenzoic acid (PABA) in the nutrition of animals and microorganisms. While the

status of PABA per se as a member of the B vitamin group has been questioned (Hogan and Kamm, 1943; Sevag *et al.*, 1946) the evidence which has accrued in its favor is impressive. Analogous with the established members of the water-soluble group, it is widely distributed in nature and is either synthesized by microorganisms or required for their growth. That PABA might be an essential nutrilite for fungi was predicted first by Woods (1940) following the demonstration of its ability to overcome inhibition by sulfonamide. This hypothesis was confirmed by Rubbo and Gillespie (1940) with the finding that PABA was required for the growth of *Clostridium acetobutylicum* and by its actual isolation from yeast (Blanchard, 1941). The number of species which have been found to require an external source of PABA now has grown to about fifteen (Woods, 1947).

Whether the essentiality of PABA resides solely in its being a constituent of the folic acid molecule remains to be ascertained; several observations which have been summarized by Woods (1947) lead away from this conclusion. Based upon (a) the additional requirements of certain organisms when grown in the presence of sulfa compounds, and (b) the decreased need for PABA in the presence of other substances, notably methionine and adenine, it is believed that the function of PABA also is associated with the formation of amino acids and purine bases (Lampen *et al.*, 1946, 1947; Woods, 1947).

PABA is produced by bacteria (Eppright and Williams, 1946; Landy *et al.*, 1943b) yeasts (Lampen *et al.*, 1945; Lewis, 1944), and molds (Hartelius, 1946) cultivated on various synthetic substrates from which the factor has been omitted. Landy *et al.* (1943b) showed that of thirty widely different organisms, PABA appeared in measurable amounts in all cultures capable of initiating growth in the basal media. The quantities formed ranged from 0.003 to 0.241 μg./ml. Similar concentrations have been found in culture liquors of *Mycobacterium tuberculosis* (Eppright and Williams, 1946). In both of these reports it is emphasized that the majority of the PABA of bacterial cultures was located outside the cells.

Although variations between and within species are prominent, yeasts (Table VII) are exceptionally potent in this substance and are probably the richest natural source of PABA. Of the various types examined, commercial bakers' and brewers' strains are comparatively high while the species grown on wood sugar stillage are low.

Since the better synthetic media which have been employed result in yields of the same order of magnitude as molasses and other natural substrates (Lampen *et al.*, 1945), it appears that neither precursors nor absorption are major factors in determining cell potencies. In fact

TABLE VII
p-Aminobenzoic Acid Content of Yeasts

	Dry cells, µg./g.	References
Hansenula suaveolens	16	132
Mycotorula lipolytica	31	132
Saccharomyces cerevisiae	7.8–95	133
Torula utilis	17–21	132, 157
Commercial foil yeast	28–53	133
Commercial bakers' yeast	22–175	133
Commercial brewers' yeast	9.3–102	138, 155

much of the PABA synthesized by yeast is released into the medium. Lampen *et al.* (1945) and Lewis *et al.* (1944) found from about 70 to 98% of that fabricated was excreted, the cell-free liquors in the former study increasing from 0.25 to 6.2 µg./ml.

Relatively few molds have been examined for their ability to produce PABA, but positive evidence has been secured with *Aspergillus niger* (Hartelius, 1946) and with most strains of *Neurospora* (Tatum and Beadle, 1942).

In contrast to many natural products, PABA exists in yeast cells predominantly in a water-extractable form (Lampen *et al.*, 1945; Lewis, 1942). The small amount of bound PABA which can be demonstrated may comprise that in folic acid and in such similar peptides as described by Ratner *et al.* (1946).

2. *Factors Influencing PABA Synthesis*

The inhibition of growth by sulfonamide has been ascribed to its interference with metabolic reactions involving PABA. If true, with those organisms capable of PABA synthesis, accelerated activity tending to overcome the inhibition might be anticipated. On this point, however, experimental data are contradictory. With yeast (Landy and Dicken, 1942a), *gonococci* (Landy and Gerstung, 1945) and *Staphylococcus aureus* (Landy *et al.*, 1943a; Spink *et al.*, 1944) acclimatization to sulfa compounds is accompanied by the increased production of PABA. Nevertheless, Lemberg *et al.* (1946) following similar procedures were unable to demonstrate any greater synthesis by *Escherichia coli* although an arylamine, largely anthranilic acid, appeared earlier in cultures containing sulfathiazole. Sevag and Green (1944) also present evidence to show that PABA formation is not directly related to sulfonamide resistance.

Iron may be required for the synthesis of PABA according to Lewis (1944) since production by *Torula utilis* decreased from 60 to only 3

μg./g. when the medium lacked iron. Eppright and Williams (1946) found that fluoride but not cyanide effectively retarded PABA formation by *Saccharomyces cerevisiae.*

VIII. Pyridoxine

The studies of Snell *et al.* in 1942 indicated that pyridoxine which had been synthesized in 1939 (Stiller *et al.*) was only one of a group of naturally-occurring compounds possessing vitamin B_6 activity. This conclusion was reached after it was observed that the pyridoxine content of various natural materials was insufficient to account for their growth-promoting activity for certain lactic acid bacteria. The unusually high response of such substances, tentatively termed "pseudopyridoxine" was shown subsequently to be due to pyridoxal and pyridoxamine which are formed, respectively, through the oxidation and amination of pyridoxine (Harris *et al.*, 1944; Snell, 1944b). Based upon the differential response of *Saccharomyces cerevisiae, Lactobacillus casei,* and *Streptococcus fecalis* to these compounds their measurement in various products is now possible (Snell, 1945). Further studies recently conducted with both microorganisms and animals have resulted in the disclosure that pyridoxal phosphate is the coenzyme in transamination (Lichstein *et al.*, 1945; Schlenk and Snell, 1945), decarboxylation (Lichstein *et al.*, 1945), and possibly carboxylation (Lyman *et al.*, 1946) reactions.

Microorganisms, as may be seen from the data in Table VIII, are appreciably better sources of pyridoxine than higher plant and animal products. Yeasts are particularly rich with brewers' yeast somewhat superior to bakers' yeast. Provided that the slightly greater potency of anaerobically propagated yeast is reproducible, it may be attributable to a greater absorption under anaerobic conditions. Lewis *et al.* (1944) have reported that *Torula utilis* absorbs some pyridoxine from the medium and similar observations have been made with lactic acid bacteria (Bohonos *et al.*, 1942). Another contributing factor to cell potencies is apparent from the data of Thompson (1942) who found that while the total synthesis of pyridoxine by *Aerobacter aerogenes* was nearly equivalent under aerobic and anaerobic conditions, less excretion took place anaerobically.

Microorganisms appear to contain all of the forms of vitamin B_6 which have thus far been described. Snell concluded from assays of yeast extract that pyridoxamine was the predominant form there, although pyridoxal was present (Snell, 1945). *Neurospora,* likewise, contains both pyridoxine and "pseudopyridoxine" (Stokes *et al.*, 1943). Pyridoxal phosphate is much less active to the assay organisms than the other members of the group but becomes active following acid hydrolysis

TABLE VIII

Pyridoxine Content of Microorganisms and Culture Liquors

	Dry cells, μg./g.	Total synthesized in cells and medium, μg./g. dry cells	References
Bacteria			
Aerobacter aerogenes (aerobic)	6.8	26.8	315
Aerobacter aerogenes (anaerobic)	18	18.23	315
Serratia marcescens	11	34	315
Pseudomonas fluorescens	5.7	75.7	315
Proteus vulgaris	6.8	16.4	315
Clostridium butylicum	6.2	23.2	315
Yeasts			
Torula utilis	35	.....	157
Commercial bakers' yeast	16[1]–65	.....	15, 27, 224, 245
Commercial brewers' yeast	25–100	.....	15, 27, 172, 245, 270, 292
Molds			
Neurospora sitophila	9.1–13.7	.....	293
Penicillium chrysogenum	23	.....	301

[1] Calculated to a dry substance basis assuming 30% initial solids.

and apparently constitutes a substantial portion of the bound pyridoxin of yeast and other natural products (Rabinowitz and Snell, 1947). The existence of still other forms, one labile to acid and the other requiring more rigorous hydrolysis procedures (Melnick *et al.*, 1945) also have been hypothesized.

The formation of pyridoxine and pyridoxal phosphate is accomplished by various organisms cultivated on synthetic media free of the vitamin B_6 group (Bellamy *et al.*, 1945; Lewis, 1944; Stokes *et al.*, 1943). Although the mechanism involved in the synthesis of pyridoxine, pyridoxal, and pyridoxamine has not been elucidated, it has been demonstrated that growing cultures of organisms which are capable of utilizing these compounds convert them to the coenzyme. In addition, resting cells of *Streptococcus fecalis* convert pyridoxamine to codecarboxylase when provided with pyruvate (Bellamy *et al.*, 1945) as receptor for the amino group.

Little attention has been given to factors affecting the degree of pyridoxine synthesis by microorganisms. Snell, and Snell and Guirard (1944a, 1943) reported that the requirement of *S. fecalis* for pyridoxine is diminished by supplementing the medium with alanine. One explana-

tion proposed for this specific effect of alanine is that it is a precursor of pyridoxine (Snell and Guirard, 1943). Opposed to this theory, however, are the facts that (a) the basal media upon which the effect is demonstrable contain some alanine (Snell, 1944a) and (b) codecarboxylase apparently is not formed when alanine is substituted for pyridoxine (Bellamy *et al.*, 1945).

With a pyridoxine-less mutant of *Neurospora*, Stokes *et al.* (1943) reported that pyridoxine, while not produced under the usual conditions, was formed when the medium was adjusted to pH 5.8 or above and ammonium salts were supplied. Since pyridoxine was shown to be present in these *Neurospora* cells, it suggests that the biochemical defect in such cultures is circumvented by the selection of culture conditions which either permit a particular reaction to function or obviate its necessity. With *Torula utilis* propagated on synthetic medium to which varying levels of iron were added, it was found that maximum synthesis of pyridoxine occurred when the concentration of iron was 0.1 p.p.m. which was suboptimal for growth (Lewis, 1944). Specific inhibitors for enzymes involved in fermentation did not retard the formation of pyridoxine by *Saccharomyces cerevisiae* (Eppright and Williams, 1946), indicating that its function in metabolism may be confined to the reactions discussed above. The pyridoxine content of cells grown on galactose was unusually high whereas on xylose little or none was synthesized (Eppright and Williams, 1946).

IX. Inositol

Although *m*-inositol has been known for many years, its biological importance was unrecognized until 1928 when Eastcott isolated and identified it as a member of Wildiers's "bios." Despite this early and auspicious linkage with the water-soluble group and more recent confirmation of vitamin activity, the occurrence and quantitative production of inositol by microorganisms has received little attention. The lack of suitable analytical methods, recently obviated by the development of microbiological procedures, has been one of the main reasons for the paucity of data on the distribution of inositol.

In nature, inositol occurs in stable complexes such as the lipositols and phytin which are resistant to the commonly-employed hydrolytic procedures. This may explain in part the wide variations which have been noted in the content of bacteria and yeasts (Table IX). Because of the difficulty in liberating inositol, the values given especially for bacteria wherein enzyme digestion was used should be considered minimal.

TABLE IX

Inositol Content of Microorganisms

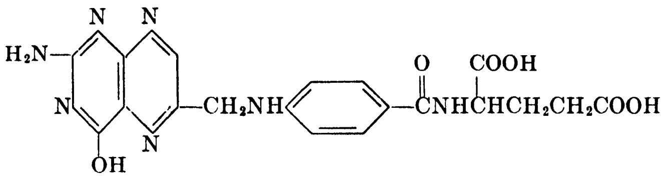
	Dry cells, µg./g.	References
Bacteria		
Aerobacter aerogenes (aerobic)	1400	315
Aerobacter aerogenes (anaerobic)	1600	315
Serratia marcescens	1600	315
Pseudomonas fluorescens	1700	315
Proteus vulgaris	1000	315
Clostridium butylicum	870	315
Yeasts		
Torula utilis	3400–3600	157
Brewers' yeast	2700–5000	157, 347

The limited data do not permit drawing conclusions regarding factors influencing the inositol content of microorganisms. Apparently some absorption is accomplished by yeast since Eastcott (1928) remarked that culturing in a medium devoid of inositol reduced the content to one-half that from an inositol-rich medium. According to Lewis (1944) an iron deficiency decreases by 25 to 50% the inositol synthesis by *Torula utilis*. *T. utilis* from a sucrose-inorganic salts medium contained from 600 to 2600 µg. inositol/g. dry cells depending upon the amount of iron supplied. When cultured in various fruit juices, the same species contained 2500–2850 µg./g. (Lewis *et al.*, 1944). That these values may be low is suggested by assays performed later employing more vigorous hydrolysis. Beadle and Horowitz using the *Neurospora* assay and refluxing the samples for 2 hours in 3% sulfuric acid obtained values of 3400 and 3600 µg./g. respectively, for yeasts propagated on molasses and prune juice (Lewis *et al.*, 1947). A sample of brewers' yeast which assayed 2700 µg./g. following mild hydrolysis was found to contain 4800 µg./g. after refluxing for 6 hours in 18% HCl (Lewis *et al.*, 1947). Woolley's values (1941) of 5000 µg./g. for brewers' yeast are in good agreement.

X. Folic Acid

Earlier reviews have dealt specifically with the various factors belonging to the folic acid group (Pfiffner and Hogan, 1946), and generally with vitamin deficiencies following the administration of sulfonamides (Daft and Sebrell, 1945). These should be consulted to provide a background for the present discussion. Much of the confusion in evidence at the time of these publications relative to the chemical nature, biological properties, and nomenclature of the various substances pos-

sessing folic acid activity has since been resolved with the determination of structure and synthesis of the liver *Lactobacillus casei* factor (pteroylglutamic acid) (Angier *et al.*, 1945).



Pteroylglutamic Acid (Liver *L. casei* factor)

In yeast and a fermentation product conjugated forms of pteroylglutamic acid occur which have been identified respectively, as pteroylheptaglutamic acid (Pfiffner *et al.*, 1946, 1947) and pteroyltriglutamic acid (Hutchings *et al.*, 1946b). Aside from these, evidence has accumulated which points toward the existence in products of microbiological origin of still other conjugates or biologically active compounds related to pteroylglutamic acid (Keresztesy *et al.*, 1943; Mills *et al.*, 1944; Ratner *et al.*, 1946).

1. *Content of Microorganisms*

Shown in Table X are the folic acid potencies of various microorganisms. Considerable attention has been given to yeasts because of their commercial availability and relatively high content, although several bacteria and lower fungi also have been examined. Conspicuous are the high potencies of certain species with respect to this vitamin and, compared with other B vitamins, a more pronounced variation in content. It should be mentioned that some of the variation is more apparent than real and results undoubtedly from the failure to completely liberate bound folic acid. This is especially true of the values given for bacteria since this investigation (Thompson, 1942) was carried out prior to the development of improved extraction procedures. While some organisms are low in folic acid content all but a few are capable of its synthesis and in only one recorded instance, that in which lactic acid bacteria were cultivated with thymine replacing folic acid (Stokes, 1944) have bacteria devoid of this substance been obtained.

Among the yeasts *Saccharomyces* species grown under both commercial and experimental conditions and *Candida arborea* are excellent sources of folic acid. Inherent differences in synthetic ability between species are indicated in the data of Agarwal *et al.* (1947). They obtained the following average values for *Saccharomyces cerevisiae*, *Candida arborea*, *Torula utilis*, and *Oidium lactis* when each was grown on four different molasses media: 20.4, 15.9, 12.5, and 7.2 μg./g. respectively.

TABLE X

Folic Acid Content of Microorganisms

	Dry cells µg./g.	References
Bacteria		
Aerobacter aerogenes (aerobic)	2.8[1]	315
Aerobacter aerogenes (anaerobic)	1.0[1]	315
Clostridium butylicium	0.5[1]	315
Proteus vulgaris	4.2[1]	315
Pseudomonas fluorescens	1.8[1]	315
Serratia marcescens	3.2[1]	315
Yeasts		
Candida arborea	14.8–19.6	3, 272
Hansenula suaveolens	1.7	132
Mycotorula lipolytica	3.1	132
Oidium lactis	5.6–14.8	3, 272
Saccharomyces cerevisiae	19.1–35.6	3, 272
Torula utilis	3.8–31.2	3, 132, 272
Commercial yeasts	15–80	162
Molds		
Penicillium chrysogenum	13.4–14.6	200
Penicillium notatum	3.6–4.5	200

[1] Values calculated assuming that the standard employed was folic acid of 20% purity.

Environmental factors affecting folic acid formation have received little attention. Since approximately equivalent yields have been secured with *Torula utilis* grown on synthetic (Lewis, 1944) and molasses media (Agarwal *et al.*, 1947) it is probable that absorption from the medium is not an important factor in determining cell contents. Nevertheless, more than fourfold variations in commercial yeast preparations were noted (Lillie and Briggs, 1947) which suggests the existence of predetermining factors. Singh *et al.* (1947) have found in most instances that yeasts were decreased in folic acid content when the rate of aeration was favorable to high yields of cells. Supplementing the medium with iron (Lewis, 1944) or sulfite (Eppright and Williams, 1946) stimulated, whereas cultivation in saturated camphor or with galactose as the source of carbon lowered (Eppright and Williams, 1946) the synthesis of folic acid by yeasts. Three yeasts, *Mycotorula lipolytica, Torula utilis,* and *Hansenula suaveolens* grown on wood sugar stillage were abnormally low in content (Kurth and Cheldelin, 1946).

Whereas with yeasts, high aeration rates lower folic acid potencies, anaerobic conditions retard production by bacteria. *Clostridium butylicum* and anaerobically-cultured *Aerobacter aerogenes* were significantly lower in folic acid than *A. aerogenes* and other bacteria grown

aerobically. A large part of the vitamin elaborated by *A. aerogenes* under aerobic conditions was found in the medium (Thompson, 1942). It might be construed from this that synthesis by bacteria is enhanced by aerobic growth while the metabolic requirement is not increased, resulting in excretion.

2. *Biosynthesis of Folic Acid*

An extensive survey of microorganisms for their ability to synthesize folic acid was conducted by Burkholder *et al.* (1945). Bacteria were cultivated on a medium composed of casein with supplementary amino acids, dextrose, salts, and vitamins other than folic acid. For the yeasts and molds the medium contained asparagin, glucose, salts and vitamins exclusive of folic acid. All cultures were aerated by shaking. Folic acid was liberated prior to assay by subjecting the whole cultures to the action of conjugase from chick pancreas.

Group	No. tested	Folic acid produced, μg./ml.
Bacteria	42	0.03–0.69
Yeasts	369	0.01–0.36
Lower fungi	84	0.002–0.096

From these data it would appear that bacteria are superior to yeasts and lower fungi as producers of folic acid; however, yeast cells (Table X) are generally richer in folic acid than bacteria. Presumably yeasts tend to retain the vitamin while most bacteria excrete it. From the results of Thompson *et al.* (1942) it is seen that from 50 to 88% of the total folic acid produced was located in the medium. On the other hand the SLR factor which can replace folic acid for *Streptococcus fecalis* is stored almost completely in the cells of enterococci (Stokes and Larsen, 1945).

Conjugase treatment applied to bacterial cultures does not result in a large release of folic acid in a manner corresponding to the treatment of yeasts and yeast extract (Burkholder *et al.*, 1945). In general bacterial folic acids, therefore, may not be conjugated, or they may be conjugated differently than in *Corynebacterium* (see below).

Data given by Burkholder *et al.* for synthesis by *Aerobacter aerogenes* are substantially higher than that found by Wright and Skeggs (1944) for the same organisms but are similar to those for *Clostridium acidi urici* (Barker and Peterson, 1944), *Escherichia coli* (Miller, 1944) and *Mycobacterium tuberculosis* (Bird, 1947). Cultures of the latter three organisms ranged from 0.024 to 0.05 μg. folic acid/ml. However, in an undefined medium fermented with a strain of *Corynebacterium*, synthesis about one hundred fold greater than the average for bacteria (Burkholder

et al., 1945) has been secured (Hutchings *et al.*, 1946a). This compound termed "fermentation folic acid" and shown to be pteroyltriglutamic acid has activity comparable to pteroylglutamic acid for *L. casei*, chicks (Jukes and Stokstad, 1947) and probably other animals (Spies, 1946; Totter, 1946).

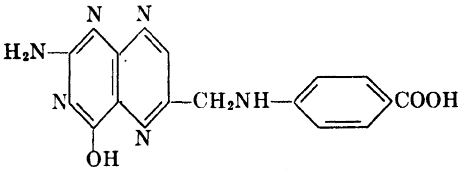
3. Production from Precursors

It has been shown by Burkholder *et al.* (1945) that certain bacteria are capable, not only of synthesizing folic acid, but also of releasing it from bound form in yeast and yeast extract. Such liberation requires according to Pfiffner *et al.* (1946) a carboxypeptidase which is found rather widely distributed among bacteria and markedly active in *Bacillus subtilis*, *Bacillus vulgatus*, *Serratia marcescens*, and an unidentified organism isolated from the intestines of a fowl. When grown on a medium containing 2% yeast extract, and 4% dextrose and having initially about 0.06 μg. free and 1.04 μg. total vitamin/ml., these organisms increased the free folic acid concentration to 0.48 to 1.67 μg./ml. An incubation period of 3 days was optimum for the conversion (Burkholder *et al.*, 1945).

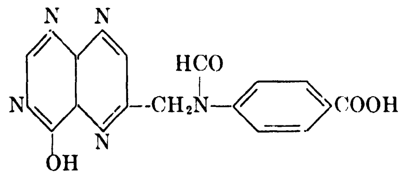
In the case of other B vitamins, *e.g.*, thiamine or pantothenic acid, it has been shown that certain microorganisms are limited in their ability to fabricate the molecule until provided with certain assimilable fragments thereof. A similar situation is indicated with folic acid from the limited data that have been compiled with intermediates thus far available. Mills *et al.* (1944) found when *Mycobacterium tuberculosis* was propagated on a synthetic medium to which *p*-aminobenzoic acid was added, chick growth factors of the folic acid type (vitamins B_{10} and B_{11}) were increased in yield. These results are readily explainable by the incorporation of *p*-aminobenzoic acid into these compounds, probably in this instance conjugates having folic acid activity. Likewise, Sarrett (1947) has observed that *Lactobacillus arabinosus* but not *L. casei* can effect the formation of folic acid when supplied with an adequate amount of *p*-aminobenzoic acid. From 5 to 15% of the PABA added was recovered as folic acid following a 70-hour incubation period.

The production of pteroylglutamic acid by microorganisms given an exogenous supply of *p*-aminobenzoyl glutamic acid, the appropriate pterin or pteroic acid should contribute toward unfolding the mechanism of biosynthesis. Pteroic acid (molecule lacking glutamic acid) has been found active in promoting growth of *S. fecalis* but not *L. casei* (Waller *et al.*, 1946). In this respect as well as structurally it resembles the SLR factor first described by Keresztesy *et al.* (1943) and Stokes and Larsen (1945). This factor which is synthesized by *Rhizopus nigricans* and

which has been characterized and termed rhizopterin by the Merck group (Wolf *et al.*, 1947) may be looked upon as a formylated pteroic acid.



Pteroic Acid



Rhizopterin

While the above are utilized by certain organisms, leucopterin and isoxanthopterin have been found inactive for *Aerobacter aerogenes* (Wright and Skeggs, 1944) and xanthopterin, convertible to folic acid by surviving rat liver (Wright *et al.*, 1944), actually inhibits folic acid elaboration by this organism.

Sulfa compounds were detected by Miller (1944) to retard the production of folic acid. According to Lampen and coworkers this is due to the interference by such compounds with the incorporation of *p*-aminobenzoic acid into pteroylglutamic acid (1946, 1947).

XI. Ascorbic Acid

Although the presence of ascorbic acid in fungi would not be unexpected in view of its wide distribution and abundance among higher plants, little conclusive evidence has been published until recently. By staining with silver nitrate, the specificity of which is questionable, the occurrence of this vitamin in numerous microorganisms has been indicated (Bourne and Allen, 1935). Confirmation of its presence, however, through animal assay or isolation, for the most part, has been lacking. While too early to draw any conclusions regarding its universal presence in microorganisms, it has been established definitely that some are capable of its synthesis.

By determining reducing activity Lewis (1939) investigated the production of ascorbic acid by several molds cultured on potato medium and Knop's solution. He observed that *Aspergillus niger*, *Cephalothecium roseum*, and *Poecilomyces* sp. increased the reducing activity of the potato substrate two- to threefold during an incubation period of 22 days. In Knop's solution after 17 days' incubation, wet mycelia

of *Aspergillus niger*, *Aspergillus flavipes*, *Cephalothecium roseum* and *Penicillium ramosus* contained respectively 1.38, 0.72, 1.39 and 0.34 mg./g. of reducing substances calculated as ascorbic acid. Most of the vitamin on the latter medium was intracellular although a small quantity appeared in the cell-free liquor.

Various *Aspergilli*, *Penicillia*, and *Mucor* species were investigated by Cuffaro (1940) who secured positive evidence only with *Mucor rosea*. The maximum yield which accumulated in the sorbose-nutrient salts medium was equivalent in reducing activity to 0.72% ascorbic acid. Gottlieb and Gilligan (1946) were unable to demonstrate the presence of ascorbic acid in mycelia of *Alternaria, Thielavia, Rhizoctonia, Helminthosporium*, and *Fusarium*.

By means of titration with 2,6-dichlorophenolindophenol Bernhauer *et al.* (1936) followed the formation of ascorbic acid by *Aspergillus niger* grown under various conditions. Highest yields (up to 13.9 mg. per 100 ml. of medium) were obtained with sucrose, sorbitol, glycerol, and inositol and were correlated with the concentration of carbohydrate employed. These observations with regard to *Aspergillus niger* were confirmed and extended by Geiger-Huber and Galli (1945). The reducing substance produced was identified with ascorbic acid both by preparation of the 2,4-dinitrophenylhydrazine derivative and by animal assay. Their culture liquors contained from 1 to 5 mg./100 ml.

With *Aspergillus niger* (Bernhauer *et al.*, 1936; Galli, 1946; Geiger-Huber and Galli, 1945) and *Mucor rosea* (Cuffaro, 1940) there is general agreement that relatively long incubation periods (6–22 days), certain minerals, high sugar concentrations, a pH of 2.0 to 3.8, adequate nitrogen source and a slightly elevated temperature are conducive to ascorbic acid production. Ketogulonic acid may be an intermediate in this fermentation (Galli 1946).

Few bacteria have been examined for their ability to elaborate ascorbic acid. Busing and Peters (1940), however, have shown that it is formed by *Bacillus prodigiosus* when grown on xylose. During a 12-day incubation period the reducing activity of the medium, as ascorbic acid, increased about 13 mg. %. Animal feeding trials substantiated the conclusion that at least a portion of the substance produced was active biologically.

Although yeasts can absorb ascorbic acid from extracts of natural products (Trufanov and Ennatskaya, 1944), they are of low potency (Boas-Fixen and Roscoe, 1940) and probably cannot fabricate the molecule. However, Lunde and Lie (1938) found marine algae to be a fairly good source of ascorbic acid, ranging from 5 to 140 mg./100 g. wet substance.

XII. Ergosterol

Ergosterol, long known to be a component of the lipide fraction of microorganisms, was first isolated by Tanret (1889, 1890) from ergot. Gerard (1892, 1895a and b, 1898) at about the same time, found it widely distributed among fungi. With the discovery that, upon irradiation, ergosterol was converted to an antirachitic substance (vitamin D_2, calciferol) this compound assumed increasing importance. The physiology and biochemistry of ergosterol has been discussed at some length by Bills (1935).

In microorganisms, ergosterol occurs principally in bound form. The palmitate ester has been isolated from several strains of *Penicillia* (Oxford and Raistrick, 1933) and more recently from *Aspergillus fumigatus* (Weiland and Prelog, 1947). Characterization of the bound form from yeast, although attempted, has been unsuccessful (Maguigan and Walker, 1940).

1. Ergosterol in Bacteria

The quantitative distribution of ergosterol in bacteria and other microorganisms is summarized in Table XI. Few bacteria have been examined quantitatively. *Mycobacterium tuberculosis*, an organism distinctive for its high lipide content, reported to be devoid of this provitamin (Anderson 1932; Anderson *et al.*, 1931; Prickett *et al.*, 1930) was later found to contain it (Hecht, 1935). On the other hand, *Bacillus butyricus* has been found to contain 0.49% ergosterol.

2. Ergosterol in Molds

Numerous molds have been investigated and almost without exception they have been shown to contain ergosterol. While molds have not been considered an important commercial source of the provitamin, industrial utilization in the form of by-product mycelia from some of the recently-developed mold fermentations would not be unexpected. For example, Zook *et al.* (1944) isolated ergosterol in crystalline form equivalent to 1% of the dry, surface-grown mycelium of *Penicillium notatum*. Cavallito (1944) confirmed the above observation with regard to surface cultures of this organism, but noted only traces in the mycelium of submerged cultures. Some strains of *P. notatum* are productive under submerged growth conditions, however, since Savard and Grant (1946) found 0.22% ergosterol in commercially-propagated mycelium from strain X-1612.

From a survey of numerous molds employing surface growth, ergosterol synthesis was found to be governed by the particular strain as well as by the cultural conditions. With *Aspergillus fischeri* (Wenck *et al.*,

TABLE XI

Ergosterol Content of Microorganisms

	Dry cells, %	References
Bacteria		
Bacillus butyricus	0.49	101
Yeasts		
Blastomyces dermatitidis	0.24	214
Endomyces hordie	0.2	26
Endomyces fibuliger	0.2	26
Endomyces lindneri	0.2	26
Monilia albicans	0.4	214
Mycoderma sp.	0.2	26
Nadsonia fulvescens	0.3	26
Saccharomyces carlsbergensis	2.0	26
Saccharomyces pastorianus	0.3	26
Torula pulcherrima	0.2	101
Torula utilis	0.5	199
Zygosaccharomyces chevalieri	0.2	26
Zygosaccharomyces mandshuricus	0.2	26
Zygosaccharomyces mellaleri	0.54	101
Commercial debittered brewers' yeast	0.18–0.34	199
Commercial brewers' strain (aerobic)	1.4	26
Commercial primary grown yeast	0.3–0.8	26, 199
Commercial distillers' strain	0.3	26
Commercial pressed yeast	0.4–2.7	87, 88
Molds		
Penicillium aurantio-bruneum	0.18	130
Penicillium aurantio-griseum	0.5	209
Penicillium biourgeanum	0.02	209
Penicillium brevi-compactum	0.5	209
Penicillium chrysogenum	1.0–1.1	48
Penicillium citrinum	1.1–1.3	48
Penicillium expansum	0.14	221
Penicillium glaucum	0.75	101
Penicillium grisio-bruneum	0.04	209
Penicillium notatum (surface growth)	1.0–1.1	48, 359
Penicillium notatum (submerged growth)	0.22	360
Penicillium puberulum	0.13	29
Penicillium zanthinellum	0.06	221
Pullularis (Dematium) pullulans	0.29	101
Aspergillus fischeri	2.23	332
Aspergillus niger	0.14	101
Aspergillus oryzae	0.26	221

1935) the potency increased with the carbohydrate supply. The highest yield, 2.23%, was secured in a medium composed of 20% glucose, 0.5% urea, and 0.68% potassium acid phosphate. The nitrogen source also

was a factor, urea being most suitable when calcium carbonate was present and nitrate when it was omitted.

3. *Synthesis by Yeasts*

Despite the fact that yeast has been one of the principal sources of provitamin D for many years, the various factors affecting its synthesis have not been clearly defined. As with the molds both strain variations and culture conditions have been implicated. At one time, according to Bills and Cox (1929), bakers' yeast was utilized as a commercial source of ergosterol. With the discovery that *Saccharomyces carlsbergensis* was characteristically a richer source, this species was adopted commercially.

It has been reasoned by several investigators (Heiduschka and Lindner, 1929; Maguigan and Walker, 1940; Smedley-MacLean, 1928; Smedley-MacLean and Thomas, 1920) that ergosterol, being a lipide, should be increased in cells by cultural practices which favor lipide synthesis. However, Terroine *et al.* (1927) were unable to substantiate this theory, and Massengale and coworkers (1932) concluded from extensive studies that ergosterol synthesis, while not correlated with lipide or protein production or with the carbohydrate reserve in the cells, did show a relationship with the particular carbohydrate used. They classified sugars insofar as their ability to promote synthesis of ergosterol was concerned into the following three groups: (a) xylose, mannitol, α-methyl glucoside, lactose, melibiose, and melizitose, (b) galactose, mannose, fructose, and glucose, and (c) sucrose and maltose. Carbohydrates in group (a) gave cells containing 5% total lipide and 0.5% ergosterol, those in group (b) 1% total lipide and 0.5% ergosterol, and those in group (c) 1.1 to 2.2% lipide and 0.7–0.8% ergosterol. Peculiarly, raffinose, which yeasts hydrolyze to fructose and nonassimilable melibiose, supported a somewhat lower cell crop but the cells contained 1.2% ergosterol. Similarly with regard to the influence of carbohydrate source, Ramaswamy and Sreenivasaya (1946) reported that yeast recovered from maltose fermentations was richer in ergosterol than that from sucrose. Ammonium sulfate also enhanced its formation slightly.

Aerobic cultivation appears to be a prime requisite for ergosterol production by yeasts. This point, about which there is general agreement, is exemplified by the observation of Bills *et al.* (1930) that brewers' yeast normally low in ergosterol could produce up to 1.4% when propagated aerobically. In addition to aerobic conditions Prickett *et al.* (1930) recommended neutral or weakly alkaline conditions. Likewise, Zorkoczy (1942) found that both total lipide and ergosterol were higher in alkaline media whereas the latter was absent in cells propagated at

pH 5.0. It was the opinion of this investigator that conditions which favored the breakdown of yeast total lipides led to the accumulation of ergosterol.

Ascorbic acid when added to a sucrose-phosphate medium heavily seeded with bakers' yeast was observed by Maguigan and Walker to enhance ergosterol formation (1940). Since the medium was actively aerated, the effect of ascorbic acid is not readily explainable. The nitrogen source and supply seemingly play a role in ergosterol synthesis also, since casein hydrolyzate and certain amino acids adversely affected synthesis when added to the above sucrose-phosphate medium.

Gordon has described a process for enriching yeasts in ergosterol which involves restricting the nitrogen supply and aerating in the presence of nontoxic oxidizing agents (1936). As suitable oxidizing agents, such compounds as persulfate, percarbonate, peracetate, perphosphate, hydroquinone, indigo carmine, methylene blue, and peroxides of potassium, sodium, and calcium were claimed. When yeast was cultivated aerobically for nine hours at 92°F. with methylene blue present and with the nitrogen supplement restricted and supplied only during the first half of the fermentation, the resulting cells contained 1.19% ergosterol compared with 0.4% for the control. A further improvement was patented by Gordon (1942) wherein the above process was modified by increasing the temperature to 98°F. for the second half of the fermentation cycle and adding during this stage ethanol or ethanol-lactic acid mixtures. By this procedure cells containing as much as 2.7% ergosterol were produced.

XIII. Vitamin K

The first indication that microorganisms might contain Vitamin K (antihemorrhagic vitamin), although not recognized as such, was in the work of Anderson and Newman (1933) with tubercule bacilli. They isolated a fat-soluble pigment from *Mycobacterium tuberculosis* and identified it as phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) which is closely related to synthetic vitamin K (2-methyl-1, 4-naphthoquinone) and since has been shown to have slight antihemorrhagic activity (Almquist and Klose, 1939). In 1935 Almquist and Stokstad (1935) observed that fish meal and rice bran, when stored under conditions favorable to bacterial activity, increased substantially in Vitamin K potency. This was attributed to the synthetic capacities of microorganisms, an hypothesis which was confirmed subsequently by pure culture studies with several species of bacteria (Almquist *et al.*, 1938). The investigations of Dam *et al.* (1941) and Orla-Jensen *et al.* (1941) along with the above make it clear that the elaboration of Vitamin K by

microorganisms is fairly common. The antihemorrhagic principle produced by bacteria has been characterized as vitamin K_2 (McKee *et al.*, 1939; Thayer *et al.*, 1939).

Of various organisms explored, highest potencies have been attained by cells of *Escherichia coli* (Dam *et al.*, 1941). Synthesis by this organism proceeded about equally as well on a synthetic medium of asparagin, citrate, glucose, and salts as on more complex media containing casein hydrolyzate and autolyzed yeast. Under the best conditions *E. coli* cells on a dry basis assayed 1300 to 1600 Dam-Glavind units per gram (equivalent to about 150 to 200 µg. vitamin K_2/g.). Cells of *Bacterium bifidum*, *Streptococcus fecium*, and *Microbacterium lacticum* were approximately one-fifth as active as *E. coli*. The greater proportion of the vitamin appeared to be retained within the cells. Not all organisms can perform the synthesis, however, since *Pseudomonas aeruginosa* (Almquist *et al.*, 1938), *Lactobacillus acidolphilus*, and yeast (Dam, 1942) have been found to be extremely low or devoid of vitamin K.

That Vitamin K is important in the nutrition of certain bacteria is demonstrated by its growth-promoting ability for Johne's bacillus (Woolley and McCarter, 1940). *Mycobacterium phlei* cells have long been employed as a supplement to the medium for culturing Johne's bacillus and thus the activity of vitamin K for this organism indicates that at least one of the stimulatory factors in such cells is due to vitamin K or closely-related compounds. Aside from the above evidence relative to its role in microbial nutrition, Woolley (1945) has observed that a derivative of vitamin K (2–3 dichloro-1, 4-naphthoquinone) is inhibitory to various species. Only with yeast, however, could the inhibition be reversed by the addition of 2 methyl-1, 4-naphthoquinone. While these findings are in a sense analogous to sulfonamide-*p*-aminobenzoic acid relationships, which suggested an essential role for the latter in nutrition, competitive inhibition is not as concise and further studies will be needed to determine the extent of the similarity. Dam *et al.* (1940) were unable to demonstrate that vitamin K influenced either growth or respiration of yeasts.

XIV. Carotenoids

Carotenoids, fat-soluble pigments of the polyene type, are widely distributed among microorganisms. Some have been related to photosynthetic reactions which are common to the purple sulfur bacteria, while others, principally α-, β-, and γ-carotene are of especial interest in view of their vitamin A activity. Whereas vitamin A has not been found, by far the greater number of fungi which produce carotenoids synthesize some β-carotene. Little is known about the other carotenoids present

in microorganisms, largely owing to their complex nature as well as to the lack of suitable methods for their isolation and identification.

Bacteria which have been reported to produce carotenoids include members of the following genera: *Staphylococcus*, *Corynebacterium*, *Mycobacterium*, *Sarcina*, and *Bacterium* (Ingraham, 1936). However, these substances do not appear to be a product of either anaerobes or facultative organisms cultured anaerobically (Ingraham and Baumann, 1934).

Mycobacterium phlei, *Corynebacterium carotenum*, and an unidentified diphtheroid have been found to contain from 0.35 to 0.58 mg. of carotenoid pigments per gram of cells (Ingraham 1935, 1936; Ingraham and Baumann, 1928). At least ten pigments including α- and β-carotene, cryptoxanthin, lutein, and zeoxanthin have been demonstrated in the carotenoid fraction of one of these organisms, *Myco. phlei* (Ingraham, 1935, 1936). When cultured in mineral oil, *Myco. lacticola* formed about 4-μg. of total carotenoid per ml., about one-fifth of which resembled β-carotene.

Among the yeasts, the *Rhodotorula* group are prolific producers of carotenoids and this characteristic serves to distinguish them from the other nonsporogenous yeasts. From *Rhodotorula rubra*, β-carotene and torulene have been identified (Lederer, 1934) and another species yielded in addition an astacin-like compound (Lederer, 1933). *Rhodotorula sanniei*, according to Fromageot and Tchang (1938a) formed at least eight pigments including β-carotene (10 μg./g.) and torulene (147 μg./g.). By biological assay *Rhodotorula glutinis* was found to contain from 5 to 10 I.U. of vitamin A activity (Zirplel, 1941). Some but not all species of *Sporobolomyces*, likewise, synthesize β-carotene as well as other carotenoid pigments (Lederer, 1934).

Of the lower fungi, *Phycomyces blakesleeanus* appears to be the outstanding producer of β-carotene with cells of this organism propagated on synthetic medium having been reported to attain potencies in the neighborhood of 0.25% (Schopfer, 1935). Carotene formation was enhanced by adding asparagin and by exposing cultures to blue or violet light.

In general, the formation and decomposition of carotene pigments by microorganisms parallels lipide storage and utilization. Glycerol, which promotes fat synthesis, is, for example, one of the most favorable carbon sources for carotenoid production. Many of the lower alcohols, notably isopropyl, stimulate, whereas β-ionone, phytol, and even vitamin A are ineffective (Baumann *et al.*, 1933; Ingraham and Baumann, 1934; Ingraham and Steenbock, 1935; Schopfer, 1943). Iron which is essential to the formation of certain pigments likewise does not influence carotenoid synthesis (Zirplel, 1941).

XV. Vitamins in Fermentation Products

Industries which employ as raw materials agricultural commodities frequently encounter serious waste disposal problems. In the fermentation industry these problems are especially challenging because large volumes of waste liquors with low solids but high B.O.D. content are produced. Residual solids from fermentations are comprised largely of the noncarbohydrate portion of the original mash constituents along with cells of the microorganisms involved and their nonvolatile products. Through research on both the biochemical and engineering aspects of waste utilization, the industry has developed processes for converting fermentation residues into valuable feed constituents. Recent developments in this field have been reviewed by Boruff (1947).

Following the alcoholic fermentation of grain mashes and distillation of the alcohol, there remains a residual liquor, "whole stillage," which contains from 5 to 7% solids. Whole stillage is passed over screens and the coarser material thus separated is pressed to remove water further and is dried in rotary driers. This fraction which constitutes approximately one-half of the total stillage solids is known as Distillers' Dried Grains or Light Grains. The material passing through the screens, "thin stillage" is evaporated to a sirup and dried on drum driers. This product known as Dried Solubles is an excellent source of protein and vitamins. For some purposes, thin solubles sirup is mixed with Distillers' Grains and dried to give a product termed Distillers' Dried Grains with Solubles or Dark Grains. Representative vitamin analyses for these and other fermentation residues are contained in Table XII. Of the final potency of distillers' by-products feeds, about two-thirds of the riboflavin, over one-half of the choline, and a small fraction of the niacin are derived from the yeast (Bauernfeind 1944a, 1944b). During the processing, thiamine and pantothenic acid, although known to be produced by yeast, are actually lost probably owing to the high temperatures reached in cooking the mash and the acidic condition of the residues during drying.

The recognition of the high riboflavin content of waste liquors from the butanol fermentation has been discussed earlier. In contrast to ethyl alcohol fermentations wherein only grain residues are totally recovered, riboflavin concentrates are prepared from the butanol fermentation of both molasses and grain. More recently, the fermentation has been adapted to whey to produce riboflavin concentrates as well as neutral solvents (Meade *et al.*, 1945).

The vitamin and protein content of waste liquors from the submerged penicillin fermentation have been examined by Tanner *et al.* (1945).

TABLE XII

Vitamin Content of Fermentation Residues

Fermentation	μg./g. dry substance							
	Thiamine	Riboflavin	Pantothenic acid	Niacin	Pyridoxine	Biotin	Choline	References
Ethanol								
Corn: solubles	6–9	15–20	29–36	140–160	8–10	2.0–2.4	6000–7000	19
Light grains	1.5	2–3	....	30–40	....	....	1500–2000	
Grains with solubles	3–4	7–10	....	70–90	....	....	4000–5000	
Wheat: solubles	4.2–10	13–15	44–46.5	210–220	....	....	....	20
Light grains	1.3–1.5	2.9–15	6.3–7.8	72–87				
Grains with solubles	3.1–5.9	2.9–4.1	26	150				
Rye: solubles	3.5–4.0	13.2–14.0	33–35	48–52	....	....	....	20
Light grains	1.6–1.9	3.6–3.8	5.5–7.0	18–19				
Grains with solubles	3.0	8.9	34.4	21.0				
Granular wheat flour: solubles	5.3–6.0	10.9–11.5	23.7–24.2	80–81	....	....	....	20
Light grains	2.2–2.6	3.4–4.5	4.4–5.9	42.5–46.8				
Grains with solubles	4.5	8.8	18.7	68.5				
Butanol								
Molasses residue	15	60	300	....	30	....	....	[1]
Penicillin								
Mycelium (*P. chrysogenum*)	2.6	39.8–47.5	150–212	107–150	20.9–24.6	0.57–1.5	....	200, 301
Mycelium-free residue	....	7.3–29	592–805	232–333	82–144	0.6–0.8	....	301
Mycelium (*P. notatum*)	6.9	20	88	180	....	0.9	....	200
2,3 Butanediol								
Wheat (*A. polymyxa*)	....	6.0	....	153	....	....	....	2
Limed Wheat Solubles (*A. aerogenes*)	....	19	19	121	....	....	....	266a

[1] Technical sales bulletin, Commercial Solvents Corporation.

Nearly a tenfold increase in pantothenic acid and a doubling of the pyridoxine concentration were noted in the course of this fermentation. Riboflavin, niacin, and biotin levels remained essentially unchanged. In processing the mash for penicillin recovery the mycelium is first separated and may be dried directly for use in feeds. After extraction of the penicillin, the residual liquor may be evaporated and dried with or without the mycelium. Feeding tests have shown no toxicities (Newell *et al.*, 1947; Tanner *et al.*, 1945).

One of the accomplishments of wartime research was the development of two fermentation processes for the production of 2,3-butanediol, one using *Aerobacillus polymyxa*, the other *Aerobacter aerogenes*. Adams and Leslie (1946) found that in *A. polymyxa* fermentations, riboflavin in the mash was increased from 60 to 106%, averaging about 80%. About 40% of the niacin was destroyed. Biosynthesis coupled with solids losses due to fermentation increases the riboflavin and niacin potencies of the grain solids, respectively, about 5.4 and 1.8 times their original content in wheat. Solubles prepared from the fermentation of wheat with *A. aerogenes* appear to be comparable to Distillers' Solubles (Seagram Research Report, 1944).

In most fermented foods, microorganisms supplement nutritional value through their elaboration of vitamins. Cheese may be taken as an example, although the number of others which might be mentioned for the purpose of illustration is manifold. Burkholder *et al.* (1943) found that thiamine, riboflavin, biotin, and niacin were increased in the surface layers of Camembert, Brie, Liederkrantz, and Limburger cheeses during ripening probably as a result of microbial activity. Aged Swiss and American types were observed by Day and Darby (1936) to contain more riboflavin than cream cheese wherein curing is negligible (6, 8, and 1.4 μg./g. respectively). Appreciable enhancement of pantothenic acid, niacin, and biotin during curing also have been reported by others (Sullivan *et al.*, 1943).

XVI. Summary

On the basis of their sporadic distribution in microorganisms as well as their general lack of essentiality, ascorbic acid and the fat-soluble vitamins and provitamins are readily separable from the regularly accepted members of the B complex. Since analytical methods for the former are neither as sensitive nor as specific as for the B vitamins, it might be argued that their occurrence is more extensive than presently indicated. However, it is difficult to conceive that, aside from the few species which require fat-soluble vitamins, all others including the

exceptionally fastidious types are capable of sufficient synthesis to satisfy possible growth requirements.

A further quantitative difference can be drawn between the two groups of vitamins mentioned above in that, where the fat-soluble vitamins and ascorbic acid are produced they are synthesized in significantly greater amounts. In support of this distinction, the profuse formation of β-carotene by *Phycomyces*, ergosterol by various species, and ascorbic acid by *Aspergillus niger* may be cited.

Contrasted with the fat-soluble factors and ascorbic acid, vitamins of the B group are universally distributed in microorganisms and occur there at a level appreciably above that commonly found in other biological specimens. Likewise, the majority of microbial species are capable of synthesizing these substances in many instances by making use of only the simpler forms of carbon and nitrogen.

While numerous bacteria, yeasts, and molds are capable of a marked degree of vitamin elaboration, yeasts usually attain highest cell potencies because of an added ability to absorb and retain vitamins within their cells. Only *p*-aminobenzoic acid is excreted in substantial amounts by the yeasts which have been studied, whereas bacteria and molds release the greater proportion of that fabricated.

In view of the fact that B vitamins have now been definitely associated as coenzymes in both anabolic and catabolic processes, it becomes of interest to attempt a correlation of these functions with vitamin formation and content. Though data are necessarily limited, there are suggestions of such a relationship. For example, thiamine, niacin, and pantothenic acid which are involved in fermentative mechanisms are generally either synthesized or absorbed more actively by anaerobically-propagated cells. On the other hand folic acid, biotin, *p*-aminobenzoic acid, and pyridoxine which participate in anabolic reactions generally are produced in greater amounts by aerobically-propagated cells. This is in accord with the enhanced assimilative activity under aerobic conditions. Exceptions such as the unusual production of pantothenic acid by *Penicillium chrysogenum* and thiamine by *Azotobacter chroococcum* under strongly aerobic conditions, however, serve to emphasize the difficulties inherent in attempting to differentiate between vitamin requirements for anabolic and catabolic reactions.

With the exception of synthesis from precursors, the production of extraordinary amounts of vitamins in most instances is associated with abnormal environmental conditions. For example, ergosterol formation proceeds at a maximum rate only when nitrogen is restricted and strongly aerobic conditions prevail. Similarly, *Candida* species elaborate ribo-

flavin only when iron levels are adjusted below that which is optimum for growth. The resulting formation of vitamins under such conditions may occur because the organism resorts to alternative metabolic pathways. The accumulation of vitamins also is akin to the blocking of synthesis in certain mutants and suggests approaches which might be applied on a broader basis to increase the production of nutrilites. However, the utilization of antivitamins to promote synthesis has not met with marked success.

Investigations on the quantitative synthesis of vitamins by microorganisms has been limited both with reference to the number of species and to the influence of conditions. It is apparent, nevertheless, that this approach is capable of providing much information of both theoretical and practical significance.

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The B Vitamins as Plant Hormones

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CONTENTS

I. Introduction

It has been known for many years that vitamins of the B group occur richly in the higher plants, in leaves, seeds, and in other organs. The

discovery in 1934 by Schopfer and by Burgeff that one member of the B group, thiamine, is an essential growth factor for a particular fungus initiated a mass of work by many investigators which has resulted in our present clear picture of the functioning of the vitamins in regulation of the growth of microorganisms. Similarly with the demonstration that riboflavin is a component of an enzyme (Warburg and Christian, 1932) the biochemical role of vitamins in a wide variety of organisms has gradually unfolded. With regard to the functioning of vitamins of the B group in the higher plants, however, a more complex situation exists than in the microorganisms or in the higher animals. Unlike the latter, the higher plant is in general able to synthesize its own vitamins, so that elucidation of the part played by vitamins by simple depletion experiments is not possible. A partial understanding of vitamin economy in the higher plant and of the basic and general role of vitamins in the plant has been made possible only by application of plant tissue culture techniques to the problem. These studies have revealed that certain of the B vitamins function in the plant as hormones, synthesized in one portion of the plant and translocated to other portions where they are needed in growth and development. Thus while the plant as a whole is autotrophic with respect to the B vitamins, the individual organs taken separately differ widely in their ability to synthesize particular members of the B complex. This review will summarize the present knowledge concerning the hormonal role of the B vitamins in the higher plant and will, it is hoped, by indicating the points at which our present information is deficient, stimulate further investigation in this field. The substances to be considered in this review include thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, *p*-aminobenzoic acid, and the purines, especially adenine. So little is known concerning the physiological roles of folic acid, inositol, and choline in the higher plant as to make discussion of them at present unprofitable.

II. B Vitamins as Root Growth Factors

1. *Requirements of Isolated Roots*

It is possible to grow pure cultures of the isolated roots of higher plants provided that the nutrient medium in which they are placed supplies not only those materials which the root normally obtains from the soil but also those materials with which the root normally is supplied by the aerial portions of the plant. The general technique used in isolated root culture is to germinate seeds aseptically, and, when the seedling roots have attained a length of one to several centimeters, to excise the growing

tips and transfer them to nutrient solution contained in flasks or Petri dishes. If an appropriate nutrient solution has been selected the tips continue to grow at approximately the rate obtaining for roots attached to the intact plant. After a period of one to several weeks, the growing tip or tips of the cultured roots may be again excised and transferred to fresh medium. This procedure may be continued indefinitely in the appropriate nutrient, and White (1934) has in fact maintained isolated tomato roots in culture for upwards of 490 passages. With some species, as the tomato (White, 1934), each root makes numerous branches which may be cultured so that clones representing individual original plants may be built up. With other roots, as the pea (Bonner and Addicott, 1937), the branch roots cannot be cultured as normal roots and it is necessary to continue in culture only the original seedling root.

The culture of isolated roots was initiated by Robbins in 1922. Robbins, who used primarily roots of corn, and to a lesser extent pea, sunflower, and lupine, found that although in most cases the freshly excised roots grew vigorously when placed in culture in medium containing mineral salts and sugar, they nevertheless grew less in each succeeding transfer until growth ceased altogether. He suggested that "the failure of an excised root to continue growth when repeated transfers of the root tip are made at once suggests that the seedling root contains some material derived from the seed other than glucose, the mineral salts . . . , water and free oxygen which are necessary for continued growth and which cannot be synthesized in the dark in solution cultures from the material supplied." Various extracts of seeds, seedlings, plants, peptone, and yeast were not able to supply the missing factor in his experiments (Robbins, 1922b). Robbins therefore early noted the possibility that the root may obtain specific root growth factors from other portions of the plant. It is of interest to note that to this day corn roots cannot be grown in culture indefinitely and that the nature of the growth factors required by corn is still obscure.

Material progress in the study of isolated root culture was announced by P. R. White in 1934. White had the good fortune to select tomato roots for his later experiments and he showed that these may be grown indefinitely in culture in a medium containing sugar, mineral salts and 0.01% of yeast extract. The yeast extract was essential to continued growth. The nature of the growth factors contained in yeast extract and essential to growth of isolated roots was next investigated by Bonner (1937) and by Robbins and Bartley (1937a) with pea and tomato respectively. Pea roots, like tomato, could be grown indefinitely in nutrient solution containing sugar, mineral salts, and yeast extract. The yeast extract could, however, be completely replaced for several passages by

crystalline thiamine in a concentration of 0.1 mg./l. nutrient. Robbins and Bartley (1937a) similarly showed that yeast extract may be partially replaced by thiamine in the culture of tomato roots. The importance of thiamine as a growth factor for isolated tomato roots was also recognized by White (1937). Thiamine is, then, one factor required by isolated roots for their growth. It is not, however, the only factor. Isolated pea roots grown on media supplemented with thiamine alone ultimately decrease in growth rate and grow progressively thinner. The second factor required by pea roots was shown by Addicott and Bonner (1938) (see also Addicott and Devirian, 1939) to be niacin. In the presence of thiamine and niacin (0.5 mg./l.) pea roots were maintained through 52 weekly transfers with no diminution in rate. Tomato roots, on the other hand, require primarily pyridoxine in addition to thiamine (Robbins and Schmidt, 1939a, 1939b, 1939c) and with these two substances combined as growth factors tomato roots can be grown indefinitely in culture.

Thiamine, niacin, and pyridoxine are required as growth factors in the cultivation of a wide variety of isolated roots in addition to pea and tomato, and the presently available information is summarized in Table I. One species, flax, has been found which is able to make a limited growth through an indefinite number of transfers in the absence of any added growth substance. The growth of these roots is, however, increased more than 10 times by the addition of thiamine to the medium. Flax further requires only thiamine for indefinite rapid growth. White clover, like flax, is able to grow indefinitely at a slow rate in the absence of thiamine but requires niacin. Of the other 12 species which have been investigated as to requirements for growth *in vitro*, all require thiamine. Seven species including pea, clover, alfalfa, cotton, *Crepis*, *Cosmos*, and radish of the varieties tested are able to grow indefinitely on media containing thiamine and niacin as the sole added growth factors. The further addition of pyridoxine to these roots is without effect. Two species, carrot and some clones of tomato, grow at a maximum rate in the presence of thiamine plus pyridoxine, the addition of niacin being without effect on growth rate. It has been reported by Robbins and Schmidt (1939a) that at least one strain of tomato roots is able to grow indefinitely, although at an exceedingly low rate, in the presence of thiamine as the sole added growth factor, but in any case growth is greatly increased by addition of pyridoxine. Four of the species investigated require thiamine and pyridoxine as growth factors but are caused to make still faster growth by the addition of niacin. These include sunflower, some strains of tomato, *Acacia*, and *Lycopersicon pimpinellifolium*. Of the 14 species investigated only one, *Datura Stramonium*, requires all three growth factors for indefinite growth in culture.

In all of the 14 cases given in Table I, additions of further substances, such as other vitamins of the B complex, purines, pyrimidines, ascorbic acid, amino acids etc., are without effect on growth rate. In addition the combination of vitamins listed for each root elicits in every case even

TABLE I

Growth Factor Requirements of Various Species of Isolated Roots

Species	Common name	Requirement for:			Reference
		Thiamine	Niacin	Pyridoxine	
Linum usitatissimum	Flax	Stim.[1]	None	None	Bonner and Devirian, 1939
Raphanus sativus	Radish	+[2]	+	None	Bonner and Devirian, 1939
Medicago sativa	Alfalfa	+	+	None	Bonner, 1940e
Trifolium repens	White clover	Stim.	+	None	Bonner, 1940e
Gossypium hirsutum	Cotton	+	+	None	Bonner, 1940e
Crepis rubra	Crepis	+	+	None	Miller, 1940
Cosmos sulphureus	Cosmos	+	+	None	Bonner, 1941
Pisum sativum	Pea	+	+	None	Addicott and Bonner, 1938
Daucus Carota	Carrot	+	None	+	Bonner, 1940e
Lycopersicon esculentum	Tomato[3]	+	None	+	Robbins and Schmidt, 1939a, b. Bonner, 1943a
Lycopersicon esculentum	Tomato[3]	+	Stim.	+	Bonner and Devirian, 1939 Robbins, 1941
Lycopersicon pimpinellifolium		+	Stim.	+	Bonner, 1940e Robbins, 1941
Helianthus annuus	Sunflower	+	Stim.	+	Bonner, 1940e
Acacia melanoxylon	Acacia	+	Stim.	+	Bonner, 1942
Datura Stramonium	Jimson weed	+	+	+	Bonner, 1940e

[1] Factor not essential but does increase root growth.
[2] Factor essential for root development.
[3] Refers to different clones of tomato roots.

faster growth than that elicited by the optimum concentration of yeast extract. It is unlikely, therefore, that other unrecognized growth factors are needed by roots of these particular species for optimal growth *in vitro*. A possible exception to this rule is the case of the amino acid glycine, which was at one time reported by White (1939b) to replace pyridoxine as a growth factor for tomato roots. In a later paper White (1943) has, however, come to the view that glycine cannot replace pyridoxine, but may at most increase growth somewhat in the presence of thiamine, pyridoxine, and niacin. Other investigators (Bonner, 1943a; Robbins and Schmidt, 1939b) have failed to find any influence of

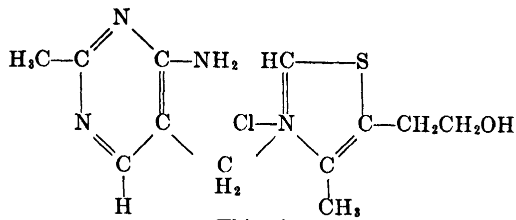
glycine on the growth of isolated tomato roots either in the presence or absence of pyridoxine.

The results presented in Table I should not convey the impression that any and all species of roots may be readily cultivated *in vitro* in a purely synthetic medium. On the contrary, the number of species whose culture *in vitro* has been unsuccessfully attempted exceeds the number for which such attempts have been successful. The roots of the *Gramineae* form a group which have resisted indefinite culture on any medium. Thus the early unsuccessful attempts of Robbins (1922a, 1922b) with corn roots were followed by unsuccessful attempts with the same plant by Fiedler (1936), by White (1932a) with roots of wheat and by the present author with roots of corn, wheat, rice, *Poa* species as well as a variety of other monocotyledenous plants (Bonner, 1940a). In all cases the roots grow well in culture immediately after excision but decrease in growth with each successive transfer, indicating that growth factors present in the seedling root are being depleted during transfer. It has not proved possible to supply this missing factor from any crude source such as extracts of yeast, seeds, seedlings, or leaves, or by any known substance or combination of substances. A report of McClary (1940) that corn roots were grown successfully through 18 transfers on a medium containing only sucrose, agar, and salts has not been confirmed (Bonner 1940a). Roots of woody species in general exhibit behavior similar to that of the grasses and to date only one woody species, *Acacia melanoxylon*, has been successfully maintained in culture (Bonner, 1942c). Not all species of dicotyledenous herbaceous plants which have been attempted have yielded to culture techniques. For example, no cucurbit root has been grown on a known nutrient medium.

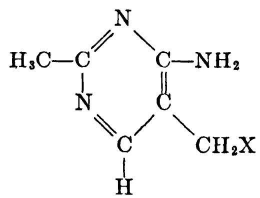
In summary, the isolated roots of a number of plant species have been cultured *in vitro* through repeated transfers on medium containing only known substances. All of these species of isolated roots require thiamine for maximum growth in culture and all but one require additionally either pyridoxine or niacin or both.

2. Specificity of Thiamine

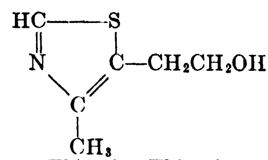
It has been shown by Robbins and Bartley (1937b) for the tomato root and by Bonner (1938a) for the pea root that in these cases the intact thiamine molecule can be quantitatively replaced as a growth factor by an equimolecular mixture of two fragments of the thiamine molecule, representing the thiazole and pyrimidine portions respectively. The two fragments, whose structure is shown on page 231, are combined in the plant into thiamine itself (Bonner and Buchman, 1938). In individual strains of tomato roots, thiamine may be replaced by the vitamin thiazole



Thiamine



Thiamine Pyrimidine
(X = reactive group as Br)



Thiamine Thiazole

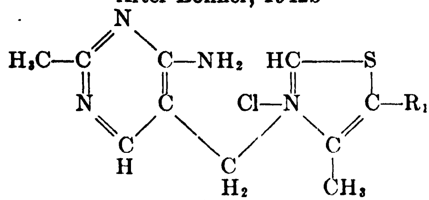
alone, addition of the pyrimidine being unnecessary (Robbins and Bartley, 1937b). It is presumed that in this case the root possesses the ability to synthesize the vitamin pyrimidine so that the compound is available for combination with added thiazole. Other strains of tomato roots, like the pea, require that both the thiazole and pyrimidine fragments of the vitamin be added to the medium (Bonner and Devirian, 1939). Flax, like the tomato strain of Robbins, requires only the thiazole fragment rather than the intact thiamine molecule (Bonner, 1940b).

The structural specificity of thiamine as a root-growth factor has been investigated only to a limited extent so far as analogues of the intact thiamine molecule are concerned. Table II summarizes the data available for excised pea roots. Analogs in which the 5-α-hydroxyethyl group is replaced by β-hydroxyethyl or hydroxypropyl side chains are not able to support continued root growth. This is true even at levels 10 times those in which thiamine is used. Similarly pyamine analogues of thiamine are ineffective. Comparable results have also been obtained with isolated tomato roots (Bonner, 1940c). A greater range of structures has been investigated using combinations of thiazole analogues with the vitamin pyrimidine or of pyrimidine analogues with the vitamin thiazole. Table III gives a summary of certain thiazole analogs tested for ability to support the growth of pea roots in the presence of the vitamin pyrimidine and of niacin. Two of the analogues, in which a β-hydroxy- or a γ-hydroxypropyl group is substituted for the α-hydroxyethyl group of the vitamin thiazole appear to be able to support growth of pea roots over considerable periods of time, although at a lesser rate than the vitamin thiazole itself given in the same concentration. This is in contrast to the behavior of the vitamin analogues containing the same two

TABLE II

The Structural Specificity of Thiamine as a Root Growth Factor for Isolated Pea Roots. All Roots Grown in a Medium Containing Inorganic Salts, Sucrose, and Niacin

After Bonner, 1942b



Thiamine

Analogue	R_1 (Thiazole ring)	Effect on growth
γ-Hydroxypropyl vitamin analogue..	$—CH_2—CH_2—CH_2OH$	No growth after four transfers
β-Hydroxypropyl vitamin analogue..	$—CH_2—CHOH—CH_3$	No growth after four transfers
α-Hydroxyethyl vitamin analogue...	$—CHOH—CH_3$	No growth after four transfers
β-Hydroxyethyl (thiamine).........	$—CH_2—CH_2OH$	Indefinite growth at an average of 90 mm./week

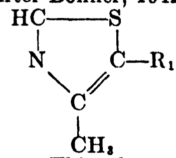
thiazoles since the vitamin analogues, as shown in Table II, are inactive. This remarkable difference between the activity of the two analogues when given as free thiazoles and when combined into a vitamin analogue is not found in other organisms where they have been tested. Thus, in *Phycomyces*, both forms of the analogues are inactive or essentially so (Bonner, 1940c). A possible explanation might be found in a thiazole sparing action of the free thiazole analogues, the analogues in some way acting to conserve the thiazole already contained in the root tip. An analogue of particular interest is β-4-methylthiazole alanine, an amino acid synthesized by Buchman and Richardson (1939), containing a 4-methylthiazole linked through the 5 position to the β carbon atom of alanine. This amino acid is active as a source of thiazole both in the pea root (Bonner and Buchman, 1938) and in the tomato (Robbins, 1940). It has been shown in the pea root that the compound is transformed *in vivo* into the vitamin thiazole (Bonner and Buchman, 1938). Although this fact shows that enzymes for the transformation of the amino acid must occur in the plant, still the amino acid is as yet unknown in nature and we have no knowledge as to any possible role which it might play.

Earlier work on the specificity of various thiazole analogues in replacing the vitamin thiazole as a growth factor for isolated pea roots (Bonner,

TABLE III

Structural Specificity of the Thiazole Portion of the Thiamine Molecule as a Growth Factor for Isolated Pea Roots. All Roots Grown in a Medium Containing Inorganic Salts, Sucrose, Niacin, and Vitamin Pyrimidine

After Bonner, 1942b



Thiazole

Thiazole analogue	R_1	Effect on growth
Vitamin thiazole	$—CH_2CH_2OH$	Indefinite growth at average of 90 mm./week
α-Hydroxyethyl vitamin thiazole	$—CHOHCH_3$	No growth after four transfers
γ-Hydroxypropyl vitamin thiazole	$—CH_2CH_2CH_2OH$	Growth continued through six transfers at an average of 55 mm./week
β-Hydroxypropyl vitamin thiazole	$—CH_2CHOHCH_3$	Growth continued through twelve transfers at an average of 48 mm./week
Vitamin thiazole alanine	$—CH_2CHNH_2COOH$	Indefinite growth at rate equal to vitamin thiazole
Imidazole analogue	....	No growth after four transfers
Vitamin thiazole-5-carboxylic acid	—COOH	No growth after four transfers

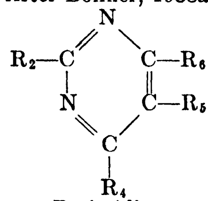
1938a) has indicated a marked lack of specificity of the thiazole portion. In contrast to the data given in Table III the earlier work was carried out with roots grown in a medium lacking niacin and hence not growing at a maximal rate. The low specificity, which contrasts strongly with the great specificity found for microorganisms (Bonner and Erickson, 1938; Robbins and Kavanagh, 1938b; Schopfer, 1934; Knight, 1937a, b; Tatum and Bell, 1946) may again be due to thiazole sparing action or other secondary effects.

The small amount of work which has been reported on the structural specificity of the pyrimidine portion of thiamine as a root growth factor indicates that even small changes in this ring abolish activity altogether. This is shown in Table IV, which refers to pea roots grown on a medium containing salts, sucrose, and vitamin thiazole. Analogues in which the

TABLE IV

Structural Specificity of the Pyrimidine Portion of the Thiamine Molecule as a Growth Factor for Isolated Pea Roots. All Roots Grown in Medium Containing Salts, Sucrose, and Vitamin Thiazole

After Bonner, 1938a



Pyrimidine

Number	R_2	R_4	R_5	R_6	Activity
1	CH_3	H	CH_2Br	NH_2	Active
2	CH_3	H	CH_2NH_2	NH_2	Active
3	CH_3	H	CH_2OEt	NH_2	Active
4	CH_3	H	CH_2NHCSH	NH_2	Active
5	CH_3	H	H	NH_2	Inactive
6	CH_3	H	CH_2COOH	NH_2	Inactive
7	CH_3	H	CH_2CONH_2	NH_2	Inactive
8	CH_3	H	CH_2OEt	OH	Inactive
9	CH_3	H	CH_2NH_2	OH	Inactive
10	CH_3	H	CH_2OH	OH	Inactive
11	OH	CH_3	CH_2OH	OH	Inactive
12	Cl	CH_3	CH_2thiazole	NH_2	Inactive

methylene group of the 5 position is substituted with Br, NH_2, or substituted amino groups are active. The substitution of OH for NH_2 at position 6 abolishes activity altogether. These results are concordant with those obtained for other organisms (Bonner and Erickson, 1938; Robbins and Kavanagh, 1938a), although it should be noted that with *Phycomyces*, replacement of the 2-methyl by an ethyl group leaves the activity of the pyrimidine substantially unaltered (Robbins and Kavanagh, 1938c).

3. *Specificity of Niacin*

The specificity of niacin as a growth factor for isolated pea roots has been investigated (Bonner, 1940d) and a summary of the results is given in Table V. Niacinamide is as active as niacin, as are also derivatives which may be readily convertible into niacin, including esters of the acid and nicotinuric acid. All other compounds tested were inactive. The specificity of niacin as a growth factor for pea roots hence parallels closely the results obtained with *Staphlococcus aureus* (Knight, 1937a, b; Landy,

TABLE V

Structural Specificity of Niacin as a Growth Factor for Isolated Pea Roots. All Roots Grown in a Medium Containing Inorganic Salts, Sucrose, and Thiamine

After Bonner, 1940d

Substance	Activity
Niacin	Fully active
Niacinamide	Fully active
Coramine (*N*-diethylamide of niacin)	Inactive
Methiodide of niacinamide	Inactive
Methyl nicotinate	Fully active
Ethyl nicotinate	Fully active
Propyl nicotinate	Fully active
Butyl nicotinate	Fully active
Nicotinuric acid	Fully active
Isonicotinic acid	Inactive
Picolinic acid	Inactive
Quinolinic acid	Inactive
Dinicotinic acid	Inactive
Ethyl nicotinylacetate	Inactive
Arecoline	Inactive
β-Aminopyridine	Inactive
Nicotino-3-nitrile	Inactive
β-Picoline	Inactive
Thiazole-5-carboxylic acid	Inactive
Amide of thiazole-5-carboxylic acid	Inactive
Benzoic acid	Inactive
Pyrazine-3-carboxylic acid	Inactive
Pyrazine-2, 3-dicarboxylic acid	Inactive

1938), dysentery bacteria (Dorfman *et al.*, 1938, 1939) and the dog (Woolley *et al.*, 1938).

4. Specificity of Pyridoxine

The specificity of pyridoxine as a growth factor for isolated tomato roots has been investigated by Robbins (1942) and a summary of the results obtained is given in Table VI. Aside from compounds readily convertible to pyridoxine only one analogue, i.e., that in which the methyl group at position 2 is replaced by an ethyl group, is active as a growth factor in this case, a result in general agreement with that obtained with the rat (Unna, 1940) and with lactic acid bacteria (Bohonos *et al.*, 1941).

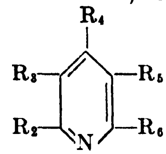
5. Deficiency Symptoms

It is evident from what has been stated above that isolated roots cultured in media deficient in a particular required growth factor exhibit the generalized deficiency symptom of reduced growth in length. More specific deficiency symptoms are, however, evident on detailed inspection. Pea roots grown on a medium containing salts, sucrose, and niacin

TABLE VI

Structural Specificity of Pyridoxine as a Growth Factor for Isolated Tomato Roots. All Roots Grown in a Medium Containing Salts, Sucrose, and Thiamine

After Robbins, 1942



Position 2	3	4	5	6	Activity
CH_3	OH	CH_2OH	CH_2OH	H	Active (pyridoxine)
CH_3	CH_3COO	CH_2CH_3COO	CH_2CH_3COO	H	Active
CH_3	OH	CH_2CH_3COO	CH_2CH_3COO	H	Active
CH_3	CH_3O	CH_2OH	CH_2OH	H	Inactive
CH_3	OH	CH_3	CH_3	H	Inactive
CH_3	$NH_2 \cdot HCl$	CH_2OH	$CH_2NH_2 \cdot HCl$	H	Inactive
CH_3	OH	$CH_2OC_2H_5$	CH_2OH	H	Inactive
CH_3	$NH_2 \cdot HCl$	$CH_2OC_2H_5$	$CH_2NH_2 \cdot HCl$	H	Inactive
CH_3	OH	CH_3	CH_2OH	H	Inactive
C_2H_5	OH	CH_2OH	CH_2OH	H	Active

but deficient in thiamine show greatly reduced meristematic activity. The cells of the meristematic region present before the onset of thiamine deficiency attain essentially the normal size and differentiation into vascular elements occurs normally (Addicott, 1939, 1941). Thiamine deficient roots consist therefore of short sections of mature root but possess only a rudimentary meristem. In niacin deficiency, on the contrary, the roots grow progressively thinner due to reduction of the number of cell columns in the meristem (Addicott, 1941) as well as to reduction in width of the individual cells. The cells of the niacin deficient roots do not elongate normally and attain only approximately 60% of the length of the cells of the normal roots. Meristematic activity is somewhat reduced in the tips of niacin deficient roots, but the reduction is less marked than in the case of thiamine deficiency (Addicott, 1941). In general it may be said that niacin deficiency results primarily in reduction of cell elongation. Pyridoxine deficiency has been histologically investigated in tomato and in *Datura* roots by Addicott (1942). Pyridoxine deficiency, like thiamine deficiency, results in lowered cell division activity in the root meristem.

6. *Relation of Isolated Roots to Other B Vitamins*

Only three of the vitamins of the B complex are required as constituents of the medium for the growth of isolated roots. The three

vitamins involved are hence evidently those which the roots in question are unable to synthesize. It is of interest to know whether the other vitamins of the B complex are synthesized by roots from the simple constituents of the medium or whether they are not needed in the root economy. It is now evident that the general answer to this question is that isolated roots synthesize those members of the B complex which they do not require as externally supplied growth factors. A simple case is that of flax roots which (as mentioned above) can grow, although at a low rate, in medium consisting only of inorganic salts and sucrose. These roots synthesize thiamine to the extent of *ca.* 0.02 γ/root/week under the culture conditions used (Bonner, 1940c). This amount is small in comparison with the 2 γ/root/week which must be added to the culture medium in order to obtain maximal growth. A similar small synthesis of thiamine was found in the case of clover roots, which, like flax, are able to grow at a low rate in the absence of added thiamine.

Biotin, which is not required as a supplement for the growth of any root yet investigated, is abundantly synthesized by roots of flax, clover, alfalfa, and tomato (Bonner, 1940e). *p*-Aminobenzoic acid is synthesized by isolated tomato roots and, incidentally, inhibition of the growth of isolated tomato roots by sulfa drugs is of a competitive nature, the inhibition by sulfathiazole, for example, being largely reversible by addition to the medium of *p*-aminobenzoic acid (Bonner, 1942f). Riboflavin is likewise synthesized by isolated roots of tomato, clover, alfalfa, *Datura*, and sunflower (Bonner, 1942d). Information on the synthesis of pantothenic acid, inositol, folic acid, and other members of the B complex by roots is not available. It seems, however, most probable that these compounds, like riboflavin, biotin, and *p*-aminobenzoic acid, are required by roots, just as by other tissues and organisms, but that the root cells possess the power of synthesizing the compounds in question from the simple constituents of the medium.

III. B Vitamins as Embryo Growth Factors

1. *Culture of Isolated Embryos*

The fact that vitamins of the B complex are stored in high concentrations in the seeds or seed parts of higher plants suggests at once that these vitamins may play a role in the embryo development, seed germination, or early growth of the plant. That this is in fact the case was first suggested by work of Kögl and Haagen-Smit (1936) with excised pea embryos from which the cotyledons had been removed and which were grown in aseptic culture. Under these conditions relatively little growth occurred on a medium containing only mineral salts and sugar. Addition of biotin to the medium resulted in considerable increases in rate of

stem growth while addition of thiamine resulted in increased growth, primarily of roots, but also to a lesser extent of the shoots. These results were extended by Bonner and Axtman (1937) who found that pantothenic acid like biotin increases the growth of stems of cotylectomized pea embryos. Additions of estrone and ascorbic acid also increased growth, although ascorbic acid is extensively synthesized by some varieties of pea embryos (Bonner and Bonner, 1938). Niacin, like thiamine increases the growth of both roots and shoots of cotylectomized pea embryos (Bonner, 1938b). Even with the optimum combination of biotin, thiamine, pantothenic acid, niacin, estrone, and ascorbic acid growth of such embryos does not approach that of plants from which cotyledons have not been removed (Bonner, 1942a) and it is evident that other as yet unknown factors are of importance in the growth of pea embryos.

The culture of immature plant embryos has long attracted attention, but detailed study of the nutritional requirements of such embryos has been made only by Van Overbeek *et al.* (1941) in the case of *Datura Stramonium*. Mature or nearly mature embryos were grown successfully in medium containing dextrose and mineral salts alone, such embryos requiring no added growth factors for further development. Embryos which were only one-third of the final size did not develop in the simple medium, however, and were only able to develop if, in addition to salts and sugar, a complex mixture of vitamins and other possibly active substances was present. This mixture included glycine, thiamine, ascorbic acid, niacin, pyridoxine, adenine, succinic acid, and pantothenic acid. The active constituents of the mixture were identified by Loo (1944) who found only pantothenic and succinic acids to be essential, the other substances being without effect. Embryos smaller than one-third of the final size were found by Van Overbeek *et al.* (1941) to develop only in still more complex media containing unknown substances derived from coconut milk. Immature embryos of other species have been grown in media containing yeast extract or fibrin digest (LaRue, 1936; White, 1932b; Brink *et al.*, 1944; Tukey, 1934).

Only a small beginning has been made in the study of the growth factor requirements of embryos during their development. It is clear that mature embryos are able to develop in simple media without additional growth factors although in some cases, as that of cotylectomized pea embryos, addition of thiamine, niacin, pantothenic acid, and biotin as well as other substances may accelerate growth. In embryos which are only moderately immature such as those of *Datura* (above) pantothenic acid is an essential factor for development. Whether the same substance is limiting in other cases is unknown. In still more immature

embryos, development *in vitro* procedes only at the expense of other as yet unknown substances which are apparently not members of the B complex. Both the vitamins such as pantothenic acid and the unknown growth factors for immature embryos would appear to constitute specific substances required for embryo development which, since they are not synthesized in the embryo, must move into the embryo from surrounding plant tissues. These materials may properly be referred to as hormones of embryo development.

2. *Cultures of Callus and Cambium*

Much attention has been paid in recent years to the culture *in vitro* of callus tissue, a subject developed and recently reviewed by Gautheret (1946) and to which White (1939a) has contributed largely. Such callus tissue may be grown *in vitro* indefinitely, the nature of the medium required depending on the tissue involved. Thus the callus tissue cultured from the spontaneous tumors of a *Nicotiana* hybrid (White, 1939a) requires no added growth factors of any kind and is apparently autonomous with respect to the B vitamins (Hildebrandt *et al.*, 1946). Callus tissues induced in normal plants by application of high concentrations of auxin require the presence of indoleacetic acid for continued development *in vitro* (Gautheret, 1946), and Morel (1946) has shown that in one case at least, *Crataegus monogyna*, pantothenic acid is likewise an essential nutrient factor. Sections of the fleshy root of carrot have been cultured by Nobécourt (1943). In contrast to the behavior of seedling carrot roots which require thiamine, the tissues of the fleshy root continue to synthesize thiamine in culture (Nobécourt, 1940). Callus of rose tissue, also cultured by Nobécourt (Nobécourt and Kofler, 1945) is reported to require addition of thiamine for maximum growth.

Other work on callus cultures which need not be reviewed here has shown that indoleacetic acid is of primary importance as a growth factor for many kinds of callus tissue (Gautheret, 1946) and has indicated that tumor tissues may differ from corresponding normal tissue in requirement for this growth factor (Skoog, 1944; DeRopp, 1947). The work of Morel and of Nobécourt does indicate, however, that (in certain cases at least) vitamins of the B complex may be required by callus tissue and it is evident that the possibility of such requirements should be considered in further work.

IV. Leaf Growth Factors

The growth of the leaf may be divided into two physiologically separate aspects, i.e., growth of the veins and growth of the mesophyll between the veins. Avery (1935) has made it probable that vein growth

in *Nicotiana* is limited by available auxin. Growth of the mesophyll on the contrary involves other growth factors including in particular the purines. Early work of Vyvyan (1924) and Went (1938a, 1938b) has shown that growth of the mesophyll of the leaves of seedlings is dependent on substances stored in the cotyledons while Gregory (1928) has produced evidence that increase in leaf area of young leaves is dependent on the already existing leaf area of the plant. While both of these facts indicate the regulation of leaf development by substances which travel within the plant, they do not indicate whether the substances involved are hormonal in nature or are merely general food materials. D. Bonner *et al.* (1939) have devised an assay for leaf-growth factors based on the increase in area of sections cut from the immature leaves of seedling radish plants. These sections are floated on a solution containing sucrose and the increase in area over a 24-hour period is measured. Extracts of pea seeds (D. Bonner *et al.*, 1939) or extracts of mature leaves (D. Bonner, 1940) when added to the nutrient solution were active in increasing the rate of expansion of such test sections. A second test for leaf-growth factor activity consisted in the aseptic culture in the dark of excised leaf primordia (approximately 1 mm. in length) of pea seedlings in solutions containing salts and sucrose. The addition of pea cotyledon (D. Bonner *et al.*, 1939) or leaf extracts (D. Bonner, 1940) greatly increased the growth of such leaves. Such cultured leaves actually attained a greater size than would have resulted had the leaf remained on the plant. In both of the assay tissues cell divisions were largely completed before excision of the test tissue and growth under influence of the active materials was largely by cell enlargement. Fractionation of the pea cotyledon extract led to the isolation of a highly active compound, hypoxanthine, and tests of pure compounds revealed that adenine also is active in promoting leaf growth. Other compounds of the B complex were inactive. Certain other compounds active in low concentrations found in the course of the work were dihydroxyphenylalanine, isolated from pea cotyledons and the further amino acids proline, asparagine, valine, glutamic acid, alanine, and leucine. So far as the growth of radish leaves is concerned the activity of pea cotyledon extract would appear to depend on varied factors in addition to the purine hypoxanthine. In the culture of excised pea leaves, however, adenine is capable of quantitatively replacing pea cotyledon extract as a leaf growth factor. Isolated whole buds of rye have been grown in culture by DeRopp (1945) and the growth of the leaves observed. Neither vitamins of the B complex nor adenine or other purines were effective in increasing the growth of leaves of this grass. The factors limiting leaf growth in rye are therefore different from those limiting leaf growth in pea or radish.

It may be concluded that while only a modest start has been made on the subject of the leaf growth factors, nevertheless special factors for control of leaf growth do exist, are made or stored in seeds and mature leaves and are required by immature leaves for expansion of the mesophyll cells. While hypoxanthine and adenine have been recognized as important in the control of leaf growth in radish and in pea it remains to be seen whether these compounds are of general significance as leaf growth hormones.

V. Stem Growth Factors

The growth of isolated stems in culture has been successfully consummated only by Loo (1945). This author cultivated isolated stem tips of asparagus (*Asparagus officinalis*) in nutrient media containing mineral salts and sugar. On this medium the stems grew actively through repeated transfers for periods of more than 1 year, provided that the cultures were maintained in diffuse light. In the dark on the contrary, growth approached zero after four transfers, owing apparently to depletion of growth factors contained in the original tip. Growth was not maintained in the dark by addition of any of the B vitamins, or by various amino acids, although growth during the first transfer was promoted by addition of succinic acid, ammonium sulfate, or aspartic acid. Excised asparagus stem tips appear, then, to synthesize in the light growth factors as yet unknown and which cannot be synthesized in the dark. The amount of light-induced synthesis must be quantitatively small, since an external supply of sucrose is essential even in light-grown cultures.

The growth of excised shoots of tomato plants in the dark has been studied by Went and D. Bonner (1943). The growth rate of such stems approaches zero after approximately 24 hours in darkness but can be increased by supplying the stems with sucrose fed through the leaves. Immersion of leaves in extracts of pea seeds, of yeast, or of coconut milk gave a further increase in growth rate above the level obtained with sucrose alone. The nature of these stem growth factors is, however, still obscure.

VI. Distribution of B Vitamins in the Plant

1. *Vegetative Plant*

It has been shown above that vitamins of the B complex are physiologically active in the plant in that they are limiting factors in the development of isolated roots, embryos, and leaves. In order to establish that these same factors act *in vivo* as hormones or correlative agents in plant

growth it is essential to discover whether they are synthesized in particular restricted areas within the organism and subsequently translocated to the organs or tissues which are unable to synthesize them and in which they are required for growth. This section and the following will consider the vitamin economy of the intact plant; the distribution, site of formation, and translocation of B vitamins in the plant.

It may be stated at the outset that the B vitamins are found in all species and in all organs of higher plants, and a brief selection of examples given in Table VII illustrates this point. Of the eight vitamins examined all were detected in all cases. Thiamine occurs in concentrations of 3–14 γ/g. in leaves, roots, and fruits, and riboflavin is found in amounts of approximately the same order of magnitude as are also pantothenic acid, pyridoxine, and folic acid. Niacin occurs in higher concentration, of the order of 50γ/g. being a usual figure. Inositol is found in very much higher concentrations, of the order of 1000–8000 γ/g. of dry material, while biotin occurs to the extent of from less than 0.1 to 0.7 γ/g. No general rules concerning the relative proportions or amounts of the several vitamins in different tissues as in leaves, roots, or fruits, are evident.

The distribution of thiamine in tomato plants grown in the greenhouse and approximately 40-cm. tall is given in Table VIII. The highest concentration of thiamine, 19.8 γ/g., is found in the shoot apex and youngest leaves. Each successively older leaf has a smaller concentration of the vitamin and a similar gradient in thiamine concentration is found in the stem. The roots are moderately high in thiamine containing 6.7 γ/g. Although the concentration decreases in each successively lower leaf still the total amount of thiamine increases to a maximum in the eighth leaf owing to the increasing weight of the older leaves. This, as will be shown below, is owing to the fact that thiamine is formed in the expanding and recently matured leaves. The thiamine distribution in maize has been investigated by Burkholder and McVeigh (1940). In this plant, as in tomato, thiamine concentrations are higher in younger than in older leaves. A similar distribution of thiamine in the pea plant has been described by Rytz (1939) and in cotton by Sukhorukov and Filippov (1940). The distribution of thiamine in woody plants is similar to that found in annuals (Burkholder and Snow, 1942) and the thiamine content of a variety of leaves and buds has been tabulated by Burkholder and McVeigh (1945a). The occurrence of thiamine as well as of riboflavin, biotin, pantothenic acid, niacin, and folic acid in oat plants of different ages has been studied by Kohler (1944). In general the vitamins tended to attain a maximum concentration in the plant as a whole at approximately the time of flowering. A vast amount of miscellaneous

TABLE VII

B Vitamin Content of Some Typical Plant Tissues

After Cheldelin and Williams, 1942

Species	Part	Vitamin content in γ/g. dry weight							
		Thiamine	Riboflavin	Niacin	Pantothenic acid	Pyridoxine	Biotin	Inositol	Folic acid
Beta vulgaris	Leaves	14	20	58	14	3.6	0.26	2000	20
Spinacea oleracea	Leaves	5.4	22	50	17	8.1	0.67	2600	23
Beta vulgaris	Roots	1.9	4.5	49	8.2	0.97	0.02	1600	3.4
Daucus Carota	Roots	4.2	4.2	22	21	10.2	0.21	4100	8.2
Brassica oleracea	Roots	3.3	8.1	86	4.6	14	0.26	5800	3.3
Pyrus Malus	Whole fruit	6.6	1.2	5.6	4.1	1.8	0.06	1600	0.55
Lycopersicon esculentum	Whole fruit	10	6.2	78	62	10	0.67	7700	13

TABLE VIII

Distribution of Various Vitamins in Tomato Plants Approximately 40-cm. Tall. Figures on Dry Weight-Basis

After Bonner, 1942e and Bonner and Dorland, 1943a, b

Part	Dry weight of part g./2 plants	Thiamine γ/g.	Pyridoxine[1] γ/g.	Riboflavin γ/g.	Pantothenic acid γ/g.
Apex	0.113	19.8	24.6	33.7	35.7
Leaf 12	0.102	16.0	43.4	35.6	23.3
11	0.172	14.8	26.9	36.6	17.6
10	0.270	13.2	24.4	35.8	19.8
9	0.356	11.4	22.7	35.6	16.6
8	0.536	10.9	22.4	32.0	15.2
7	0.516	9.4	23.6	26.0	17.0
6	0.528	8.1	20.3	24.4	14.0
5	0.392	7.8	20.0	18.6	11.3
4	0.344	7.0	20.0	16.4	14.1
3	0.242	5.7	16.1	15.2	14.9
2	0.161	4.7	14.8	11.4	
1	0.137	4.5	....	10.5	
Top 10 cm. of stem	0.310	10.0	....	6.2	20.8
Next 10 cm. of stem	0.620	4.2	....	5.7	14.4
Next 10 cm. of stem	0.572	3.0	....	4.2	9.7
Bottom 10 cm. of stem	0.662	2.4	....	2.8	8.6
Roots	1.135	6.7	13.9	10.3	29.0

[1] Separate set of plants from those used in other determinations.

information on the thiamine content of plants and plant parts of interest from the standpoint of human food value is available but is in general not informative in the present connection.

The detailed distribution of pyridoxine in the vegetative tomato plant generally parallels that of thiamine, the highest concentration being in the younger leaves and lower concentrations in the older leaves, the highest concentration being 43 γ/g. and the lowest 14 γ/g. (Table VIII, Bonner and Dorland, 1943b).

Riboflavin and pantothenic acid distributions generally parallel that of thiamine in the vegetative tomato plant, although the concentration gradient from younger to older leaves is less striking (Table VIII, Bonner and Dorland, 1943a). Although detailed analysis of the distribution of other members of the B complex in the plant is lacking, still it is known, as discussed above, that the other vitamins as well as *p*-aminobenzoic acid and adenine, occur generally in plant tissues including leaves. Thus Burkholder and McVeigh (1945a) found thiamine, riboflavin,

pyridoxine, niacin, inositol, biotin, pantothenic acid, and folic acid in leaves or buds of all of 18 species investigated while Kohler (1944) found thiamine, riboflavin, biotin, pantothenic acid, niacin, and folic acid in *Avena* plants of all ages studied. In summary, it appears probable that the vitamins of the B complex occur universally in higher plant tissues, and in so far as the matter has been looked into, the vitamins are present in highest concentration in the young leaves of the vegetative plant with a general gradient of decreasing concentration toward the older portions. The same vitamins are found also in the stems and roots.

2. Flowers, Fruits, and Seeds

The floral parts of the plant, like the vegetative parts, possess appreciable contents of B vitamins. Thus Rytz (1939) found thiamine in young buds, calyx, corolla, stamens, and pistil of pea and tulip, as is shown in Table IX, while Cravioto *et al.* (1945) found thiamine, riboflavin, and

TABLE IX

Vitamin Contents of Flowers and Floral Organs of Various Species

Species	Thiamine content, γ/g. dry weight				
	Immature buds	Calyx	Corolla	Stamens	Pistil
Pisum sativum[1]	23	21	14	13	20
Tulipa Gesneriana[1]	..	4	4	10	5

Species	Thiamine, γ/g. dry flower	B_2, γ/g. dry flower	Niacin, γ/g. dry flower
Cucurbita mexicana[2]	20.0	25.0	122
Brassica campestris[2]	4.6	1.5	73
Agave atrovirens[2]	12.2	5.6	23.2
Yucca aloifolia[2]	14.0	9.0	58.0

[1] After Rytz, 1939.
[2] After Cravioto, *et al.*, 1945.

niacin in whole flowers of various species. Thiamine as well as niacin, pantothenic acid, and riboflavin have been found in a variety of pollens by Vivino and Palmer (1944). Detailed analysis of the distribution of vitamins other than thiamine in the flower is lacking but the available data, of which a selection is given in Tables VII and X, indicates that fruits and seeds in general contain all members of the complex. Contrary to what might be expected, seeds do not appear to be generally

higher in vitamin content than the other portions of the fruit when compared on a dry weight basis (Table X).

TABLE X

Vitamin Contents of Some Typical Fruits and Seeds

After Cravioto *et al.*, 1945

Species		Vitamin content, γ/g. dry wt.		
		Thiamine	Riboflavin	Niacin
FRUITS				
Cucurbita mexicana	Squash	10.0	10.0	70
Cucumis sativus	Cucumber	6.5	8.7	39.2
Persea gratissima	Avocado	3.4	4.9	39.4
Prunus Armeniaca	Apricot	1.8	1.8	25.1
Passiflora lingularis	Passion fruit	0.5	4.7	70.2
Lycopersicon esculentum	Tomato	11.1	5.6	66.3
Citrus nobilis	Tangerine	6.6	2.0	14.5
Mangifera indica	Mango	6.9	2.4	80.5
Cucumis Melo	Melon	4.6	3.1	81.6
Citrus Aurantium	Orange	6.7	2.2	18.6
SEEDS				
Sesamum indicum	Sesame	14.2	2.4	52.3
Phaseolus vulgaris	Bean	9.2	2.0	24.2
Arachis hypogaea	Peanut	10.0	1.4	52
Cicer arientium	Chick pea	9.6	1.8	19.0
Cucurbita Pepo	Squash	3.8	2.4	34.6

The distribution of vitamins within the seed has been thoroughly studied for a number of species. Table XI, taken from Rytz (1939),

TABLE XI

Thiamine Content of Pea Seeds during Development and at Maturity

After Rytz, 1939

Organ	State of maturity	Thiamine content, γ/g. dry weight
Whole seed	Immature	19
Whole seed	Mature	15
Cotyledons	Mature, not dry	24
Cotyledons	Mature, dry	11
Embryos	Mature dry seeds	16

shows that the thiamine content of the whole pea seed reaches a maximum at maturity and before drying out of the seed. In the mature dry seed, the cotyledons contain 11 γ/g. and the embryo 16γ/g. dry weight.

TABLE XII

Distribution of Vitamins of the B Complex in Cereal Seeds

Species	Part	Concentration of vitamin, γ/g. dry weight			
		Thiamine	Riboflavin	Niacin	Reference
Avena sativa (oat)	Endosperm	11.7	1.6	9.5	McVeigh, 1944
	Root of embryo	20.6	13.7	77.5	
	Coleoptile of embryo	12.5	20.0	107	
Triticum vulgare (wheat)	Endosperm	4.3	....	.....	Pulkki and Puutula, 1941
	Embryo	13.9	....	.....	
Triticum vulgare (wheat)	Scutellum	120	15	.....	Hinton, 1942
	Embryo	12	15		
Triticum vulgare (wheat)	Endosperm	1.8	....	.....	Ward, 1943
	Scutellum	148			
	Embryo	19.1			
Secale cereale (rye)	Whole grain	2.4	....	.....	Hinton, 1944
	Scutellum	114			
	Embryo	6.9			

Table XII shows that thiamine is relatively low in concentration in the endosperm of cereal seeds, is higher in the embryo and is exceedingly high in concentration in the scutellum, concentrations of up to nearly 150 γ/g. being reported. Since the scutellum makes up approximately 1.5% of the dry weight of the seed, over one-half of the total thiamine of the seed is contained in this one tissue. Riboflavin and niacin are also present in higher concentration in the embryo than in the endosperm. The riboflavin of the endosperm is found in highest concentration in the aleurone layer according to Somers *et al.* (1945).

VII. Translocation of the B Vitamins

1. *Vegetative Plants*

That vitamins of the B complex are translocated in the plant can readily be shown by the use of the girdling technique, a standard physiological tool in the study of translocation of materials within the plant. The method of girdling which has been used primarily is one in which the living tissues of the stem or petiole are killed over a short region by a jet of steam. Translocation through the phloem is then blocked at this point although movement of water and salts through the nonliving elements of the xylem continues. Substances which are normally trans-

located through the phloem accumulate at the girdle and from the position of the accumulation the normal direction of transport of the material can be deduced. If the stems of tomato plants are girdled near the root level, thiamine accumulates rapidly in the region of stem immediately above the girdle and is gradually depleted not only from the stem below the girdle but from the entire root system of the plant (Bonner, 1942e). It would appear therefore that thiamine is normally transported from the aerial portions of the plant toward the root, a conclusion similar to that drawn by Burkholder and McVeigh (1942) from girdling experiments with woody plants. Thiamine transport in the vegetative plant occurs not only toward the root but also toward the apical growing point, as has been shown by the accumulation of thiamine below girdles made near the growing point of vegetative tomato plants (Bonner, 1942e). Since the only evidence available concerning the growth factor requirements of isolated buds (Loo, 1945) indicates that such tissue does not require added thiamine for growth *in vitro*, the physiological significance of this acropetal transport is not clear.

The synthesis of thiamine in the plant takes place in the leaves as has been shown in a variety of ways with the tomato (Bonner, 1942e). Thus the transport of thiamine through the stem toward the root occurs only in plants which possess leaves. If petioles are girdled, accumulation of thiamine occurs on the laminar side of the girdle. Thiamine formation and export of thiamine occurs most vigorously in the mature leaves whereas little or no transport occurs from young rapidly expanding leaves. These leaves must nevertheless be capable of thiamine synthesis, since they increase in total thiamine content in the absence of any detectable thiamine transport to them from the older leaves.

Pyridoxine, like thiamine, is produced in the leaves of tomato plants and is exported from the leaves to the roots (Bonner and Dorland, 1943b). Rapid accumulation of pyridoxine takes place in the stem above a basal girdle. Riboflavin, on the contrary, does not accumulate to any extent in the region of stem above a girdle and would hence appear not to be transported toward the roots (Bonner and Dorland, 1943a) while pantothenic acid accumulates only to a moderate extent. Neither riboflavin nor pantothenic acid is transported toward the apical growing point. Riboflavin is not exported from the leaves of tomato plants, and it appears that the various vegetative tissues of the tomato must all possess the ability to synthesize their own supply of this factor as has been shown above for the roots. Pantothenic acid is translocated from tomato leaves and the leaves are undoubtedly an active site of pantothenic acid synthesis. Translocation of pantothenic acid from the leaves to the roots occurs, however, only to a limited extent. In summary, of the four

vitamins whose translocation in the vegetative tomato plant has been studied, thiamine and pyridoxine, which the root is unable to synthesize and which are required as root growth substances, are formed in the leaves and vigorously transported to the roots. Riboflavin, which is synthesized by roots, appears to be synthesized by other tissues also and

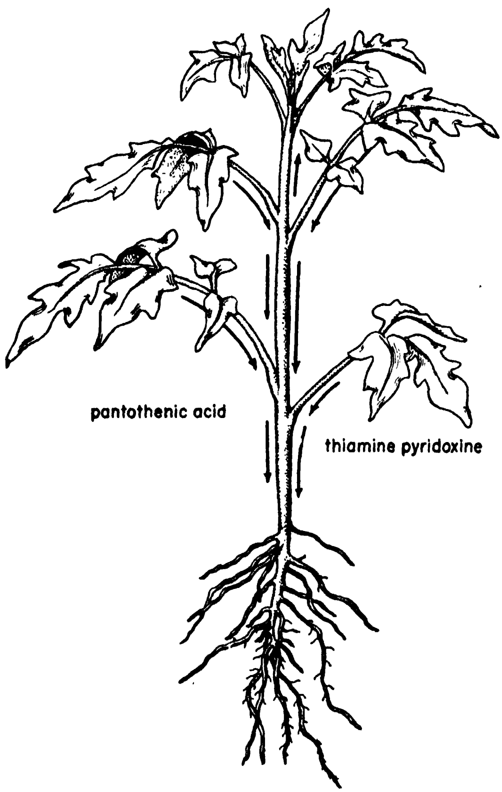


FIG. 1.—Diagrammatic representation of the directions of transport of thiamine, pyridoxine, and pantothenic acid in the tomato plant. Riboflavin is not translocated.

is not translocated appreciably, whereas pantothenic acid represents an intermediate case. This vitamin, which is not required as a root growth factor, is exported from the leaves but is translocated toward the roots only to a limited extent.

The synthesis and translocation of purines has been studied primarily with respect to the methylated purines, caffeine, and theobromine. These alkaloids as well as xanthine, hypoxanthine, guanine, and adenine occur primarily in leaves and the purines themselves are probably universal constituents of leaves. The methylated purines are distributed

in the plant as are the B vitamins, with highest concentrations being found in the youngest leaves and lower concentrations but greater total amounts in the older leaves (Weevers, 1930). In the stem the methylated purines are found only in the bark and the suspicion must be that the substances may be translocated in the phloem, although direct evidence, aside from the inconclusive work of Weevers (1930, 1932) is meager. Went (1932) found caffeine to be taken up by living cells of *Impatiens* stems and translocated polarly in the apical direction. Further studies on the role of purines as leaf growth factors should include investigations of the site of synthesis and the subsequent translocation of the substances.

The site of synthesis of other members of the B complex is as yet unknown. It may be suspected by analogy with the results already obtained that niacin may be formed in leaves, and then translocated to the roots, particularly in species whose roots are incapable of niacin synthesis. Biotin, *p*-aminobenzoic acid, the purines, and other factors which are synthesized in roots may, like riboflavin and pantothenic acid, be translocated from leaves to roots only sluggishly if at all.

2. Translocation of Vitamins into Reproductive Organs

It has been shown above that vitamins of the B complex including thiamine, pyridoxine, niacin, biotin, and pantothenic acid are essential factors in embryo and/or seedling development as judged by *in vitro* culture experiments. The vitamins supplied to the developing embryo must therefore derive either from tissues of the ovary other than the embryo or from the vegetative portions of the plant. Similarly the

TABLE XIII

Apparent Translocation of Thiamine from Glumes, Stems, and Leaves to the Fruit, during the Development of Wheat

After Geddes and Levine, 1942

Days after flowering	Dry weight per grain mg.	Thiamine content γ/g.			Thiamine content γ/plant			Total
		Fruits	Glumes etc.	Leaves and stem	Fruits	Glumes etc.	Leaves and stem	
7	3.4	7.2	3.3	3.2	0.9	1.1	4.5	6.5
10	6.5	7.6	2.8	3.1	1.9	1.0	4.2	7.0
14	12.7	6.6	1.7	2.7	3.4	0.6	3.4	7.3
17	15.3	5.9	1.5	2.4	3.6	0.5	2.9	6.9
21	19.2	5.7	1.4	2.1	4.3	0.4	2.2	7.0
24	20.7	5.2	1.1	1.8	3.9	0.3	1.8	6.0
28	23.0	5.2	0.6	1.2	4.2	0.2	1.1	5.5

vitamins stored in the cotyledons or endosperm and used by the seedling in seedling growth may derive either from synthesis *in situ* or from translocation from the leaves or other vegetative organs. The source of the thiamine stored in developing wheat grains has been considered by Geddes and Levine (1942). As the seeds increase in size and in total thiamine content, the thiamine of the glumes and rachis, stem, and leaves together decreased by an amount approximately equaling that appearing in the seeds, as is shown in Table XIII. It would seem that in wheat new synthesis of thiamine ceases at or shortly after the beginning of seed development and that filling of the seeds with thiamine is accomplished by translocation of the vitamin from the leaves and other vegetative tissues. In the pea, on the other hand, the synthesis in the vegetative plant continues during seed development so that withdrawal of thiamine toward the seed, if it occurs, is masked (Rytz, 1939).

The mechanisms by which B vitamins other than thiamine accumulate in the tissues of the fruit, seed or embryo, whether by translocation or by synthesis *in situ* have not been investigated. It would be of the greatest interest to study this aspect of the vitamin economy of the higher plant, particularly in view of the light which might then be thrown on the possible role of vitamins as controlling agents in fruit and seed development.

3. *Behavior of B Vitamins in Seeds during Germination*

Related to the question of the vitamin economy of the embryo during development is the status of the B vitamins during seed germination. In the germination of pea seeds from which the reserve of the cotyledons has been removed, thiamine is not synthesized to an appreciable degree when the plants are grown in the dark (Bonner and Greene, 1938). When the plants are grown in the light, however, major increases in thiamine occur, as shown in Table XIV. A similar requirement of light

TABLE XIV

Synthesis of Thiamine in Cotylectomized Pea Embryos Grown in Light or Dark

After Bonner and Greene, 1938

Condition	Plant part	Vitamin B_1 content, γ/part. Days after removal of cotyledons and initiation of experiment		
		0	4	8
Dark	1-cm. Root tip	15	15	18
	Apical bud and 2 leaves	28	32	32
Light	1-cm. Root tip	15	16	46
	Apical bud and 2 leaves	28	36	100

for thiamine synthesis in the seedling also obtains for germinating tomato seeds (Bonner, 1941) and for wheat (Hoffer *et al.*, 1946). Even in seeds grown in the light the seedling plant may be totally dependent on its reserve of thiamine during the initial stage of germination, as shown by the data of Table XV for black-eyed peas and lima beans. In cotton, on the other hand, some synthesis of thiamine may occur in the light even within 36 hours. Riboflavin is synthesized in the germinating seeds

TABLE XV

Synthesis of Various Vitamins in Germinating Seedlings in the Light

After Cheldelin and Lane, 1943

Vitamin	Vitamin contents, γ/g. dry weight					
	Blackeyed peas		Lima beans		Cotton	
	0 time	36 hrs.	0 time	36 hrs.	0 time	36 hrs.
Thiamine	8.5	12	5.8	4.7	3.2	4.9[1]
Riboflavin	1.5	3.9[1]	1.4	2.4[1]	2.3	2.8
Niacin	14	24[1]	11	16[1]	16	24[1]
Pantothenic acid	11	13	9	11	11	22[1]
Pyridoxine	2.0	3.3[1]	6.0	6.7	0.9	2.6[1]
Biotin	0.22	0.31	0.11	0.16[1]	0.29	0.28
Inositol	2600	4400[1]	1800	3100[1]	3400	2800
Folic acid	7.8	4.6	3.0	2.7	3.0	7.9[1]

[1] Probable significant synthesis of vitamin during the experimental period. The data given do not permit of correction for the decreased dry weight of the seed during germination.

of wheat, corn, and beans even in the dark, according to Burkholder and McVeigh (1942), and Table XVI gives data on the behavior of several vitamins in dark-germinated seeds of soybean and mung bean. Similar synthesis of riboflavin occurs during germination in the light for black-eyed peas and lima beans (Cheldelin and Lane, 1943, Table XV). Niacin is synthesized in seedlings in the dark in the case of soy bean and mung bean (Table XVI) and during the initial stages of germination in the light in black-eyed peas, lima beans, and cotton (Table XV). Pantothenic acid, pyridoxine, biotin, inositol, and folic acid are synthesized during the early germination of some species, but not in others (Tables XV and XVI). In their study of the behavior of the B vitamins in seeds of soy and mung bean germinating in the dark, Burkholder and McVeigh (1945b) found no synthesis of folic acid but large increases in riboflavin, niacin, and inositol. Only small increases in pyridoxine were found while pantothenic acid and biotin behaved differently in different cases (Table XVI).

In summary it would seem that the seed, while it may be able to synthesize a portion of the B vitamins during germination, is nevertheless in general dependent on the stored reserve for one or more members of the complex. When germination occurs in the dark this is true of thiamine but not of riboflavin or in general of niacin although the niacin requirement of pea embryos implies a lack of synthetic ability for this vitamin in the particular instance of the pea embryo. The mobilization

TABLE XVI

Behavior of B Vitamins in the Germination of Soy and Mung Beans in the Dark. Germinated for 4 days

After Burkholder and McVeigh, 1945b

Vitamin	Vitamin content, γ/g. dry weight			
	Soy bean (Peking)		Mung bean	
	Dormant	Germinated	Dormant	Germinated
Thiamine	16.2	18.8	8.2	8.4
Riboflavin	3.8	4.9[1]	3.0	14.4[1]
Pyridoxine	12.6	13.2	11.4	13.3
Niacin	21.8	31.5[1]	23.1	66.5[1]
Pantothenic acid	36.3	34.4	16.4	33.7[1]
Inositol	2436	3784[1]	1849	3649[1]
Folic acid	1.86	0.71	2.47	1.96
Biotin	0.90	1.09	0.22	0.68[1]

[1] Probable significant synthesis in total amount of vitamin per plant.

of thiamine from endosperm to embryo during germination has been demonstrated by McVeigh (1944) in *Avena sativa.* The embryo also mobilizes riboflavin and niacin, but in addition to the amounts of the two vitamins derived in this manner the tissues of the embryo appear to be able to synthesize them even in the dark. The most comprehensive study of vitamin mobilization in the germinating seedling is that of Hoffer, Alcock, and Geddes (1946) on the redistribution of these substances in wheat. Wheat seeds were allowed to germinate in the dark and the total amounts of thiamine in the endosperm and embryo were followed over an 18-day period. While total thiamine remained constant, indicating lack of new thiamine synthesis, the thiamine content of the embryo increased from an initial 11% of the total to 60% of the total after 18 days (Table XVII). This accumulation of thiamine in the embryo was attended by a corresponding loss of thiamine from the endosperm.

TABLE XVII

Translocation of Thiamine from Endosperm to Embryo during the Germination of Wheat Seeds in the Dark

After Hoffer, Alcock, and Geddes, 1946

Portion	Thiamine content γ/100 plants			
	Days incubation			
	2	4	9	18
Embryonic axis	1.4	5.8	6.5	9.6
Half of endosperm including scutellum	10.5	7.6	5.8	3.6
Brush half of endosperm	1.8	1.6	1.3	1.0
Total thiamine	13.7	14.4	13.6	14.4

VIII. B Vitamins as Growth Factors for Intact Plants

Since certain of the B vitamins are required as growth factors by roots of several species and since, as shown above, certain of the vitamins, particularly thiamine, are synthesized only slowly in the germinating seed, it is natural to inquire whether any of the B vitamins may be limiting factors in the growth of the intact green plant. It is known that most if not all species of higher plants (with the exception of saprophytes and parasites) can be grown in the light in media containing only inorganic nutrient salts. It is clear therefore that at most the further addition of vitamins might be expected to promote growth; it would not be expected that vitamins would in any case be essential to the growth of the intact plant in the way in which thiamine, pyridoxine, and niacin are required in the nutrition of isolated roots. Extensive investigation of this question has revealed that available thiamine is not a limiting factor in the growth of most species and that thiamine can become a limiting factor only in a few particular species or under particular environmental circumstances.

Evidence that selected species may respond to additions of thiamine with increased growth was first presented by Bonner and Greene (1938). Thiamine at concentrations of 0.01 to 1 mg./l. water or nutrient was supplied to sand culture- or soil-grown plants, including *Camellia* and other relatively slow growing woody species, and increases in shoot and root growth of 30 to over 100% were reported. More recent data have also confirmed the effect of thiamine on the growth of *Camellia japonica* in nutrient culture in the greenhouse (Bonner, 1945). In subsequent papers (Bonner and Greene, 1939, Bonner, 1943b) additions of thiamine were reported to increase the growth of herbaceous species grown in

nutrient culture. Thus in a series of 15 experiments *Cosmos* plants were found to produce 27% more dry weight of tops when supplied thiamine than when thiamine was omitted from the nutrient. The growth promoting effect of thiamine was obtained, however, only under conditions of relatively low temperature such as a continuous temperature of 68°F. When the plants were grown under higher temperatures, growth was greatly increased and no response to thiamine was evident. That the growth promoting effect of thiamine on *Cosmos* is in general reproducible is shown by the results of 28 experiments with *Cosmos* carried on in a cooperative survey by 15 different investigators scattered widely over the United States (Cooperative Report, 1941). Twenty-two of these experiments yielded growth increases as a result of thiamine addition, the mean increase in top dry weight amounting to 27%. The dependence of response to thiamine on environmental conditions (Bonner, 1943b) no doubt accounts for the failure of certain investigators to obtain any effects with application of thiamine to *Cosmos* (Hamner, 1940; Arnon, 1940, and others). Positive effects of thiamine have been reported for other species, as summarized in Table XVIII. Thiamine has also proved to act as a growth promoting factor for green algae in sterile culture (Van Overbeek, 1940, Ondratschek, 1940a–d, 1941a, b). In general, however, application of thiamine to cultivated species is without any apparent effect on growth. Thus, in the cooperative experiment referred to above, only two of sixteen species showed any growth response to thiamine application. Negative results of thiamine application have been reported for orange trees (Parker, Turrell, and Bonner, 1941), tomato and pea (Bonner and Greene, 1939) and for other species, of which a general summary is given in Table XIX.

It must be concluded that while thiamine may be a limiting factor in a few scattered species or under particular conditions, still availability of this substance does not in general limit plant growth. This emphasizes the hormonal nature of thiamine in the plant. Even though the thiamine supply of the tomato root, for example, is dependent on the transport from the leaves, still the leaves are apparently able to provide the roots with an ample supply of thiamine and the plant taken as a whole is thiamine autotrophic. The thiamine supply of the roots may in some cases even be so abundant as to permit of extensive loss of thiamine to the medium in which the plant is grown, as shown by West (1939) in the case of flax seedlings, the roots of which appear to excrete thiamine as well as biotin.

The effects of vitamins other than thiamine on the development of intact green plants have been but little investigated. Niacin and pyridoxine have been reported to influence growth of *Cosmos* (Bonner

and Bonner, 1940) under the same conditions of unfavorably low temperature which permit of response to thiamine. Adenine was also found to be effective in promoting growth and dry weight accumulation of *Cosmos*, leaf size in particular being affected. Niacin is ineffective in increasing

TABLE XVIII

Summary of a Portion of Experiments Showing a Growth-Promoting Effect of Thiamine Application on Growth of Various Species of Plants

Species	Common name	Reference
Agrostis tenuis	Bent grass	Bonner and Greene, 1939
Aleurites Fordii	Tung	Bonner and Greene, 1938
Allium Cepa	Onion	Borgström, 1939
Allium fistulosum	Spring onion	Borgström, 1939
Anethum graveolens	Dill	Murneek, 1941
Arbutus Unedo	Strawberry tree	Bonner and Greene, 1938
Atropa Belladonna	Belladonna	Zopf, 1940
Begonia sp.	Begonia	Fraps and Fudge, 1942
Beta vulgaris	Sugar beet	William, 1939
Bougainvillaea glabra	Bougainvillaea	Bonner and Greene, 1938
Brassica alba	White mustard	Bonner and Greene, 1939
Brassica nigra	Black mustard	Bonner and Greene, 1939
Bryophyllum degremontianum	....	Bonner and Greene, 1938
Callistephus chinensis (flat grown)	China aster	Hitchcock and Zimmerman, 1941
Camellia japonica	Camellia	Bonner and Greene, 1938 Bonner, 1945
Capsicum sp.	Ornamental pepper	Murneek, 1941
Cattleya Harrisoniae	....	Meyer, 1944
Cattleya hybrid	....	Bonner and Greene, 1939
Cedrus libani	Cedar of Lebanon	Bonner and Greene, 1939
Ceratonia Siliqua	Carob	Bonner and Greene, 1939
Correa ventricosa	....	Bonner and Greene, 1939
Cosmos sulphureus	Cosmos	Murneek, 1941 Cooperative report, 1941 Bonner, 1943b
Daphne odorata	....	Bonner and Greene, 1939
Eucalyptus ficifolia	Scarlet-flowering gum	Bonner and Greene, 1938
Lathyrus odoratus	Sweet pea	Cooperative report, 1941
Myrsine africana	....	Bonner and Greene, 1939
Poa pratensis	Kentucky blue-grass	Cooperative report, 1941
Poa trivialis	Rough blue-grass	Bonner and Greene, 1939
Prunus ilicifolia	Catalina cherry	Bonner and Greene, 1939
Ricinus communis	Castor-bean	Zopf, 1940
Rodriguezia sp.	....	Meyer, 1944
Valeriana officinalis	Valerian	Neipp, 1943
Viola tricolor	Pansy	Fraps and Fudge, 1942
Xanthium pennsylvanicum	Cocklebur	Bonner and Greene, 1939

TABLE XIX

Summary of a Portion of Experiments Showing no Effect of Thiamine Application on Growth of Various Species of Plants

Species	Common name	Reference
Agrostis tenuis	Colonial bentgrass	Clark, 1942
		Ahlgren, 1941
Antirrhinum majus	Snapdragon	Fraps and Fudge, 1942
Aster sp.	Aster	Fraps and Fudge, 1942
Aster sp.	Aster	Laurie and Kiplinger, 1941
Avena sativa	Oats	Lundegårdh and Wikén, 1943
		Templeman and Pollard, 1941
Begonia sp.	Begonia	Fraps and Fudge, 1942
Beta vulgaris	Sugar beet	Lundegårdh and Wikén, 1943
		Bouillenne and Roubaix, 1942
Beta vulgaris	Beet	Minnum, 1941a
Brassica alba	White mustard	Clark, 1942
		Arnon, 1940
		Hamner, 1940
Brassica Napobrassica	Rutabaga	Minnum, 1941a
Brassica oleracea	Cabbage	Hamner, 1940
Brassica oleracea	Cauliflower	Minnum, 1941a, b
Callistephus chinensis	China aster	Hitchcock and Zimmerman, 1941
Chilopsis linearis	Desert-willow	Donnelly, 1941
Chrysanthemum morifolium	Chrysanthemum	Swartz, 1941
		Fraps and Fudge, 1942
		Laurie and Kiplinger, 1941
		Cooperative experiment, 1941
Cineraria cruenta	Cineraria	Fraps and Fudge, 1942
Citrus sinensis	Orange	Parker *et al.*, 1941
Cucurbita Pepo	Squash	Minnum, 1941a
Dahlia pinnata	Dahlia	Fraps and Fudge, 1942
		Hamner, 1940
Dianthus Caryophyllus	Carnation	Cooperative experiment, 1941
Fraxinus velutina	....	Donnelly, 1941
Gardenia jasminoides	Gardenia	Fraps and Fudge, 1942
		Laurie and Kiplinger, 1941
Gladiolus sp.	Gladiolus	Reeves, 1942
Gleditsia triacanthos	Sweet locust	Donnelly, 1941
Gossypium hirsutum	Cotton	Woodhouse and Morris, 1942
Helianthus sp.	....	Gisiger, 1943
Helianthus sp.	....	Fraps and Fudge, 1942
Holcus Sorghum	Kafir	Myers *et al.*, 1941
Hordeum vulgare	Barley	Lundegårdh and Wikén, 1943
Juglans Hindsii	....	Donnelly, 1941
Lactuca sativa	Lettuce	Haber and Edgecombe, 1940
		Arnon, 1940

TABLE XIX.—(*Continued*)

Species	Common name	Reference
Lemna sp.	Duckweed	Gorham, 1941
Linum usitatissimum	Flax	Gisiger, 1943
Lobularia maritima	Sweet alyssum	Cooperative experiment, 1941
Lolium perenne	Perennial rye-grass	Ahlgren, 1942
Lycopersicon esculentum	Tomato	Bonner and Greene, 1939
		Templeman and Pollard, 1941
		Arnon, 1940
		Minnum, 1941a
Malus domestica	Apple	Tukey and Brase, 1941
Olea verrucosa	....	Donnelly, 1941
Oryza sativa	Rice	Minarik, 1942
Phaseolus vulgaris	Bean	Minnum, 1941a
		Gisiger, 1943
Phleum pratense	Timothy	Ahlgren, 1942
Pinus ponderosa	Yellow pine	Fowells, 1943
Pisum sativum	Pea	Bonner and Greene, 1939
Poa pratensis	Kentucky blue-grass	Ahlgren, 1941, 1942
		Laurie and Kiplinger, 1941
Poa trivialis	Rough blue-grass	Ahlgren, 1941
Prosopis juliflora	Mesquite	Donnelly, 1941
Prunus Persica	Peach	Tukey and Brase, 1941
Pyrus betulaefulia	Pear	Tukey and Brase, 1941
Raphanus sativus	Radish	Minnum, 1941a, b
		Swartz, 1941
		Hamner, 1940
Rosa multiflora	Rose	Tukey and Brase, 1941
Rosa sp.	....	Fraps and Fudge, 1942
Rosa sp.	....	Laurie and Kiplinger, 1941
Secale cereale	Rye	Lundegårdh and Wikén, 1943
Simmondsia californica	Goat-nut	Donnelly, 1941
Spirodela polyrrhiza	Duckweed	Gorham, 1945
Tagetes lucida	Marigold	Swartz, 1941
Trifolium repens	White clover	Ahlgren, 1942
Triticum aestivum	Wheat	Gisiger, 1943
Vaccinium corymbosum	Blueberry	Kramer and Schrader, 1942
Vinca sp.	Periwinkle	Cooperative experiment, 1941
Xanthium pennsylvanicum	Cocklebur	Arnon, 1940
Xanthium pennsylvanicum	Cocklebur	Hamner, 1940
Zea Mays	Corn	Gisiger, 1943
		Woodhouse and Morris, 1942
		Minnum, 1941a
		Myers *et al.*, 1941
Zinnia elegans	....	Fraps and Fudge, 1942
		Hamner, 1940

growth of tomato (Templeman and Pollard, 1941). Riboflavin has been suggested to increase growth of pot cultures of egg plant and tobacco (Dennison, 1940) but this effect has not been obtained by other investigators (Bonner, 1940c, Minnum, 1941b). Pyridoxine and pantothenic acid are ineffective in causing growth increases with radish or cauliflower (Minnum, 1941b).

A special case of vitamin requirements by intact green plants is presented by the orchids. In this group, the seeds are ordinarily germinated on nutrient media in aseptic culture and the young plants transferred to purely mineral substrate only after the attainment of a considerable size. With many forms, sugar and inorganic salts provide the essential factors for seed germination and seedling development. In other cases, however, the addition of further factors is essential to development, as has been shown by Burgeff (1934) for orchids of the *Vanda* group and by Schaffstein for *Phalaenopsis* (1938). Identification of the active factors has been made in the case of a *Cattleya* hybrid which failed to germinate on medium containing only purified sugar and salts. Addition of pyridoxine permitted good seed germination but only poor further growth, whereas addition of niacin permitted both good germination and excellent development of the seedlings (Noggle and Wynd, 1943). Thiamine has been found to increase the rate of development of seedlings of *Rodriguezia* and *Cattleya* species (Meyer, 1944).

IX. Vitamins of the B Complex as Growth Factors for Cuttings

The fact that thiamine, niacin, and pyridoxine are required as growth factors for isolated roots immediately suggests the possibility that these same substances might be of utility in promoting the growth of roots in the rooting of cuttings. It is known that the application of indoleacetic acid or other compounds of the auxin group to the basal portion of cuttings results in the initiation of root primordia in many species. The auxins are, however, inhibitory to the further development of the root, and it appears to be an essential feature of root induction by auxins that the active substance must have long disappeared before actual outgrowth of the new roots can take place (Cooper, 1938). In particular instances, auxin application to the cutting results in the formation of root primordia which fail to grow out into roots. Such a situation has been described by Went *et al.* (1938) in the *Camellia.* In a particular experiment cuttings treated with indoleacetic acid which had formed root primordia but on which no roots had grown out were retreated with thiamine (1 mg./l.). Within 24 hours the primordia present made visible growth and ultimately grew out into normal roots. Similar experiments with *Camellia,*

Dracena, laurel, lemon, *Chamaelaucium*, and other species have been reported by Warner and Went (1939). It would appear that with the particular species and individuals used, the thiamine in the cutting and available at the region of root initiation may have been insufficient to provide for the elongation of new roots. It has been stressed by Warner and Went (1939) that the promotion of root elongation by thiamine in auxin-treated cuttings appears to apply only to particular species and conditions of the tissue, and this view has been substantiated by other workers. Thus Deuber (1940) found a considerable increase of root growth in hemlock cuttings after thiamine application while no effect was evident with Norway spruce. *Fraxinus* cuttings have given a small but measurable response to thiamine (Thimann and Delisle, 1939). Small or negative effects have been obtained with thiamine treatment of cuttings by Haber and Edgecombe (1940) and Tincker and Unwin (1939). Pearse (1939) has reported increased root growth as a result of thiamine application to auxin-treated cuttings of pear, quince, apple, plum, and cherry, while Hitchcock and Zimmerman (1940) have reported that thiamine in combination with auxin increases the rooting of some species.

Pyridoxine, like thiamine, promotes the rooting of the cuttings of individual species as reported by Stoutemeyer (1940) who found nine species to respond to this vitamin. Cuttings of *Lonicera nitida* in particular rooted in response to pyridoxine additions even without preliminary application of auxin. Rooting in this species would seem to be limited primarily by available pyridoxine.

The application of biotin to stems treated with indoleacetic acid has been found to increase the number of roots formed by cuttings of etiolated pea seedlings (Went and Thimann, 1937). The effect of biotin differs from that of thiamine, which is effective only in promoting outgrowth of roots, in that biotin actually promotes the formation of root primordia. Inositol which was also tested was ineffective.

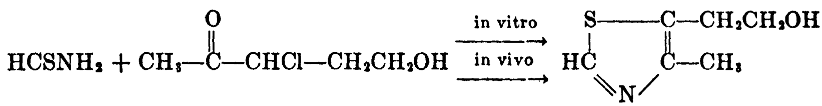
This summary shows that in particular instances the root growth substances thiamine or pyridoxine may be limiting factors in the process of root growth in cuttings. No detailed investigation as yet has attempted to correlate these effects with vitamin content or status of the cutting. Other members of the B complex and in particular niacin also remain to be investigated as factors in the rooting of cuttings.

X. Biochemistry and Metabolism of the B Vitamins

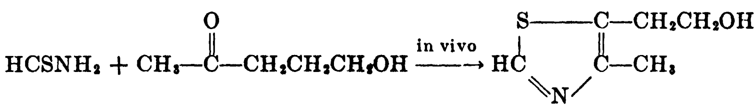
1. *Thiamine*

It has been shown above that the synthesis of thiamine may take place in roots at the expense of the free thiazole and pyrimidine moieties

and the same is true of certain microorganisms as *Phycomyces Blakesleeanus* (Bonner and Buchman, 1939b). The biosynthesis of thiamine is then resolved into the course of biosynthesis of the thiazole and pyrimidine portions of the molecule. A possible course of the biosynthesis of the thiazole portion of the thiamine molecule in the plant is suggested by experiments of Bonner and Buchman (1938) with isolated pea roots. The thiamine thiazole may be synthesized in the test tube from thioformamide and chloroacetopropyl alcohol. Isolated pea roots are able



Thioformamide Chloroacetopropyl Alcohol Thiamine Thiazole



Thioformamide Acetopropyl Alcohol Thiamine Thiazole

to utilize a mixture of these two substances to satisfy their thiazole requirement and in fact synthesize the vitamin thiazole from the mixture. Acetopropyl alcohol, unlike chloroacetopropyl, does not react spontaneously with thioformamide to form the vitamin thiazole. A mixture of thioformamide and acetopropyl alcohol can, however, also be used by pea roots to satisfy their thiazole requirement, and here again actual synthesis of thiazole appears to take place in the presence of the two components. Whether this reaction is a general reaction by which thiazole is synthesized in plants is as yet unknown. *Phycomyces* is not able to carry out the condensation while *Neurospora* can carry it out only to a limited extent (Tatum and Bell, 1946). No evidence as to the mode of biosynthesis of the pyrimidine portion is available.

Thiamine pyrophosphate is the coenzyme of the enzyme pyruvate carboxylase, as was first shown by Lohman and Schuster in the case of yeast (1937). The enzyme carboxylase occurs in some tissues of higher plants including pea roots (Horowitz and Heegaard, 1941), seeds and seedlings of cereals (Bunting and James, 1941, Bartlett, 1942), tomato seedlings (Bartlett, 1942), cucurbit seeds and fruits (Vennesland and Felsher, 1946) and various leaves (Vennesland and Felsher, 1946). In the case of the pea root the enzyme contains cocarboxylase (Horowitz and Heegaard, 1941), and cocarboxylase has also been reported from a number of other species (Tauber, 1937). We may thus picture the overall course of thiamine metabolism in the higher plants in a preliminary way as follows:

$$\left.\begin{array}{l}\text{Thiazole Precursors} \rightarrow \begin{array}{c}\text{Vitamin}\\ \text{Thiazole}\end{array}\\ \text{Pyrimidine Precursors} \rightarrow \begin{array}{c}\text{Vitamin}\\ \text{Pyrimidine}\end{array}\end{array}\right] \rightarrow \text{Thiamine} \rightarrow \text{Cocarboxylase}$$

Although the biochemical basis of thiamine action in the plant may in particular instances rest in part on the participation of cocarboxylase in pyruvate decarboxylation, still this system cannot be the only one in which thiamine is involved, since many tissues of higher plants are free of pyruvate carboxylase including spinach leaves (Bonner and Wildman, 1946) and various seeds, leaves, and fruits (Vennesland and Felsher, 1946). Oxalosuccinate carboxylase has been studied by Ochoa and Weisz-Tabori (1945) in animal tissue and found to contain cocarboxylase as a component, as does the system for oxidative decarboxylation of pyruvate by bacteria (Lipmann, 1937, 1939, 1941). In summary, the biochemical basis of thiamine activity in the plant undoubtedly resides in participation of cocarboxylase in one or more decarboxylating enzyme systems of the respiratory mechanism including possibly pyruvate carboxylase, α-ketoglutarate carboxylase, and perhaps others such as the oxalacetate carboxylase (Vennesland and Felsher, 1946).

The destruction of thiamine proceeds rapidly in cultures of *Phycomyces* by a reaction in which the thiazole portion of the molecule is inactivated with the liberation of an equivalent amount of free pyrimidine (Bonner and Buchman, 1939b).

$$\text{Thiamine} \rightarrow \begin{array}{c}\text{Inactive Thiazole}\\ \text{Breakdown Products}\end{array} + \text{Vitamin Pyrimidine}$$

The inactivation of the thiazole portion presumably proceeds by a reaction involving position 2 of the thiazole nucleus, since if the H of this position is substituted by CH_3 or NH_2 the resultant thiazole is not destroyed. It is probable that thiamine inactivation proceeds along similar lines in leaves since free uncombined pyrimidine has been found in leaves of all species investigated (Bonner, 1942b).

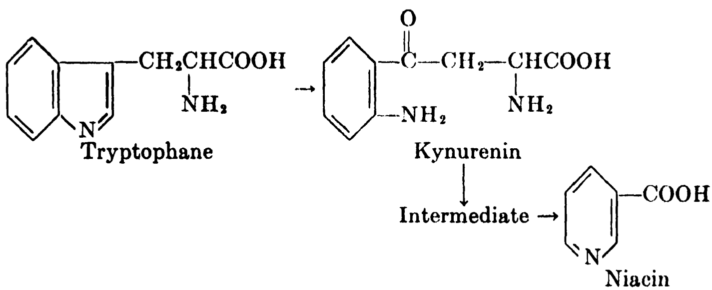
2. Riboflavin

Riboflavin functions as a component of nucleotides which are the prosthetic groups of the flavoprotein enzymes. Of the numerous flavoproteins which have been described, only one, diaphorase, which is capable of reoxidizing reduced cozymase, has been found thus far in higher plants. Lockhart (1939) has shown the presence of this enzyme in pea and bean seedlings and in potato tubers, while Okunuki (1940) has found the enzyme in pollen. Thus riboflavin appears to be in the plant as in other organisms a constituent of a respiratory enzyme, and in the particular case known, a constituent of an enzyme which acts as

an intermediary in the transport of electrons from codehydrogenases to cytochrome or other terminal oxidase systems. It is very probable that further work will reveal the presence of other flavoproteins in higher plant tissues.

3. Niacin

The biosynthesis of niacin has been studied primarily in microorganisms rather than in higher plants, and on the basis of this work as well as from investigations on niacin synthesis in the rat, it is known that the synthesis of this vitamin proceeds from tryptophane with kynurenin as an intermediate (Beadle *et al.*, 1947; Rosen *et al.*, 1946).



On the basis of the number of genetic blocks in mutant *Neurospora* strains for the synthesis of niacin from tryptophane it is clear that at least three steps are involved, one being before kynurenin and two being after kynurenin. Roots, which need an external supply of niacin, are unable to utilize tryptophane to replace the former.

Niacin is utilized in higher plants as in other organisms as a constituent of the nucleotide cozymase and possibly also of triphosphopyridine nucleotide. On the basis of the comparative growth factor requirements of microorganisms (Lwoff and Lwoff, 1937a, b) it is probable that the course of synthesis of cozymase from niacin procedes through niacinamide, niacinamide riboside and finally to the coenzyme:

Niacin → Niacinamide → Niacinamide Riboside → Cozymase

Enzymes of higher plant tissues for which cozymase is a coenzyme include malic dehydrogenase, alcohol dehydrogenase, and glutamic dehydrogenase, while a variety of other dehydrogenases must be suspected of occurring in the plant. Isocitric dehydrogenase of higher plants appears to require triphosphopyridine dinucleotide as a coenzyme (Berger and Avery, 1944).

The parsley root oxaloacetate carboxylase system of Gollub and Vennesland (1947) appears able to carry out the reversible reactions:

$$HOOCCH_2\underset{OH}{\underset{|}{C}}HCOOH \rightleftarrows HOOCCH_2\underset{O}{\underset{\|}{C}}COOH \rightleftarrows CH_3\underset{O}{\underset{\|}{C}}COOH + CO_2$$

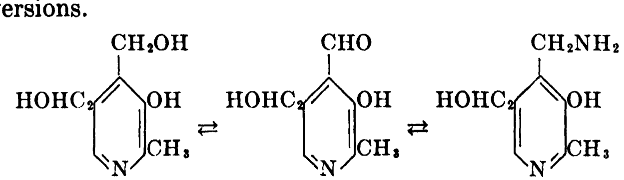
Malic Acid   Oxaloacetic Acid   Pyruvic Acid

This reaction involves the triphosphopyridine nucleotide, probably in the oxaloacetate-malate step. An interesting if little understood relationship between niacin and indoleacetic acid has recently been reported by Galston (1947). Indoleacetic acid is inhibitory to the growth of isolated asparagus roots grown in culture in the dark while niacin is without effect. Niacin in the presence of indoleacetic acid greatly enhances the inhibitory effect of the latter.

In summary, niacin functions in the plant as a constituent of cozymase and triphosphopyridine nucleotide and as such is a constituent of essential respiratory enzymes.

4. Pyridoxine

It has been found by Snell *et al.* (1942) that pyridoxine is only one of a group of similar compounds and that the different forms possess widely differing activities as growth factors for various microorganisms. Thus pyridoxal, in which the 4 position is substituted by an aldehyde group, is highly active as a growth factor for *Lactobacillus casei.* With *Streptococcus lactis* and *S. faecalis*, both pyridoxal and pyridoxamine, in which the 4 position contains a methylamino group, are active as growth factors. With *Saccharomyces Carlsbergensis* and various fungi, pyridoxal, pyridoxamine, and pyridoxine are equally active. These facts suggest that pyridoxal may be the central compound and may be converted to pyridoxamine or pyridoxine. Organisms such as *S. lactis* would then be those able to carry out interconversion of pyridoxal and pyridoxamine while those as *L. casei* would be those unable to carry out any of the interconversions.



Pyridoxine   Pyridoxal   Pyridoxamine

It has further been shown by Gunsalus *et al.* (1944a; 1944b) that pyridoxal can be phosphorylated by adenosine triphosphate in the presence of dead cells of *S. faecalis*. The resulting pyridoxal phosphate is the coenzyme of the amino acid decarboxylase of microorganisms, an enzyme which decarboxylates tyrosine, glutamic acid, arginine, glycine, ornithine, and other acids to the corresponding amines (Gunsalus and Bellamy, 1944b); Umbreit and Gunsalus, 1945; Gale and Epps, 1944).

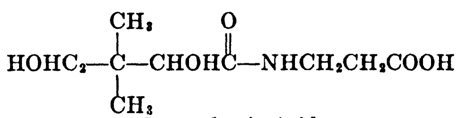
The glutamic acid decarboxylase of higher plants (Okunuki, 1937) has been shown by Schales *et al.* (1946) to have pyridoxal phosphate as a coenzyme.

Pyridoxal phosphate is also a coenzyme for the enzyme transaminase as has been shown by Lichstein *et al.* (1945) with *S. faecalis.* Transaminase has been shown to occur in higher plant tissues by Braunstein and Kritsman (1937), Virtanen and Laine (1941), Albaum and Cohen (1943), Rautanen (1946) and others. It is possible that with higher plant transaminase as with that of microorganisms, pyridoxal phosphate may act as a coenzyme.

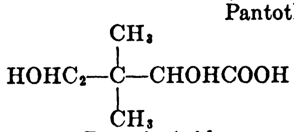
Pyridoxine is, then, active in the plant through its conversion to pyridoxal phosphate, a coenzyme of one and possibly two reactions important in N metabolism. In its possible role as a coenzyme in transamination, in particular, it may occupy a key position in the synthesis of amino acids and hence ultimately in the synthesis of the proteins of the plant.

5. *Pantothenic Acid*

Pantothenic acid, like thiamine, is made up of two moieties whose biosynthesis must be considered separately.



Pantothenic Acid



Pantoic Acid

$NH_2CH_2CH_2COOH$

β-alanine

The β-alanine moiety of pantothenic acid is alone sufficient to satisfy the pantothenic acid requirement of many yeasts (Williams and Rohrmann, 1936) which are able to synthesize the pantoic acid fragment. Beta-alanine is doubtless produced in the plant by decarboxylation of aspartic acid, an enzyme for this reaction having been found in *Rhizobium* by Virtanen and Laine (1937).

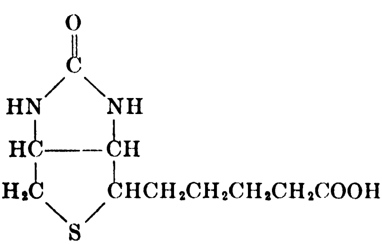
$$\underset{\text{Aspartic Acid}}{HOOC{-}CHNH_2CH_2COOH} \rightarrow \underset{\beta\text{-alanine}}{NH_2CH_2CH_2COOH} + CO_2$$

The pantoic acid moiety suffices to fulfill the pantothenic acid requirement of *Acetobacter*, which is able to synthesize the β-alanine portion (Underkofler *et al.*, 1943). Other organisms require both moieties (Tatum and Beadle, 1942) or intact pantothenic acid (Cheldelin *et al.* 1945). The reactions leading to pantoic acid synthesis are unknown.

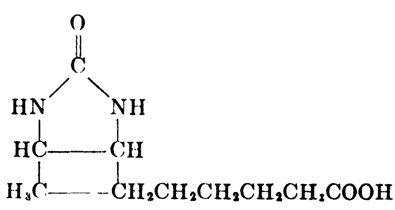
A biochemical role of pantothenic acid has been indicated by Lipmann *et al.* (1947) who has shown that this substance is a component of coenzyme A, the coenzyme of an enzyme which carries out acetylation. The role of coenzyme A appears to involve a reaction between acetate and ATP with the production of a compound having the properties of acetyl phosphate. Coenzyme A is also synthesized from pantothenic acid by the microorganism *Proteus morganii*, where it appears to function in the oxidation of pyruvate, presumably in utilization of acetate produced in oxidative decarboxylation of pyruvate (Novelli and Lipmann, 1947).

6. *Biotin*

That the course of biotin synthesis in nature is through desthiobiotin, a substance capable of replacing biotin as a growth factor for many yeasts and fungi has been shown by Tatum (1945) in experiments with two mutant strains of *Penicillium chrysogenum*. The growth factor requirement of one form was satisfied by either biotin or desthiobiotin while the second form responded to biotin alone. The second strain in addition produced a substance apparently identical with desthiobiotin which could serve as a growth factor for the first strain. Thus the strain which can utilize biotin alone is one which has lost the ability to transform desthiobiotin to biotin.



Biotin



Desthiobiotin

The addition of pimelic acid to cultures of *Phycomyces* (Bonner and Buchman, 1939a) or of other microorganisms (du Vigneaud *et al.*, 1942) greatly increases the production of biotin by these biotin autotrophic organisms. Further increases in biotin production in the case of *Phycomyces* were elicited by the addition of both pimelic acid and cysteine. Tatum (1945) in his work with *Penicillium* found that the production of desthiobiotin was increased twenty-fold by addition of pimelic acid. It is possible, therefore, that pimelic acid may be a precursor of desthiobiotin and that synthesis of biotin takes place along the following lines:

$$\text{Pimelic Acid} + \genfrac{}{}{0pt}{}{\text{Unknown}}{\text{Precursor}} \rightarrow \text{Desthiobiotin} + \text{Cysteine} \rightarrow \text{Biotin}$$

At present, however, nothing is known concerning the activity of these compounds in replacing biotin as a growth factor for higher plant tissues, as for example in embryo culture work. Lichstein and Umbreit (1947a, b) have shown that additions of biotin reactivate oxaloacetate carboxylase preparations of *Escherichia coli* which have been deactivated by maintaining the cells at pH 4 for 15–60 minutes at 37°C. A deaminase of the same organism, active on aspartate, serine, and other amino acids, may be similarly deactivated and subsequently reactivated by additions of biotin. The exact role of biotin in these enzymes as well as the possibility of biotin participation in still other enzyme systems remain to be elucidated.

7. *Adenine and Other Purines*

Older concepts of purine synthesis *in vivo* have been based on the reaction of urea with methyl glyoxal (Onslow, 1931), followed by reduction and dehydration to yield an intermediate perhaps isomeric with xanthine. Recent work shows, however, that purine synthesis, in the animal at least, is more complicated. In the formation of uric acid in the pigeon, isotopic carbon contained in CO_2, acetic acid, or glycine was incorporated in different positions in the purine nucleus (Sonne *et al.*, 1946). No evidence that urea is involved as a precursor could be obtained. It will be of interest to apply isotopic methods to the study of purine synthesis in the plant.

In the animal body adenine is oxidatively deaminated by the enzyme adenase to form hypoxanthine. Hypoxanthine is in turn attacked by the flavoprotein xanthine oxidase (which in turn contains adenine as a constituent) to yield xanthine which finally is oxidized to uric acid. Guanine similarly is oxidatively deaminated to xanthine by the enzyme guanase. These enzymes with the exception of guanase (Schittenhelm, 1909) have not been studied in higher plants and it is not known whether purine metabolism in the leaf or other plant tissue parallels that found in animal tissue. On the other hand, the fact that uric acid as well as allantoin and allantoic acid are found in plant tissues (Fosse *et al.*, 1926, 1932) suggests that purine breakdown in the plant may follow lines generally similar to those found in the animal.

The purine adenine which, as described above, is active as a leaf growth factor in pea and radish is a constituent of several varied biochemical systems in the plant. Thus adenine is a constituent of the nucleic acids of the cytoplasm and nucleus, of cozymase, of triphosphopyridine nucleotide, of the flavoproteins, and of adenosine di- and triphosphate. In the last role adenine is involved in plant metabolism, as

well as in microorganisms and in higher animal tissue, in transphosphorylation reactions such as phosphorylation of hexose (Bonner and Wildman, 1946).

XI. Summary

The green plant taken as a whole is in general able to synthesize amounts of the B vitamins adequate for its own needs and is with few exceptions independent of any external source of these chemical substances. The individual parts of the plant, however, differ widely in their ability to synthesize the several vitamins of the B complex. Since present evidence tends to indicate that these substances are required by all living tissues, this means that a tissue lacking the ability to synthesize a given vitamin must obtain its supply from other tissues of the plant. Thus roots in general lack the ability to synthesize thiamine. Thiamine is required by roots and the thiamine which they need is produced in the leaves and translocated to the root system. The amounts of thiamine concerned are small, concentrations of the order of 5–20 γ/g. of dry tissue being the usual order in the vegetative plant. Thiamine therefore fulfills the requirements demanded of a hormone in that it is synthesized in one organ and transported to another where in small amounts it brings about a physiological response, e.g., growth. The same is known to be true of pyridoxine, likewise a growth factor for roots of many species. In other species pyridoxine may not function as a root growth hormone, since in the pea, for example, the root does not require external supplies of pyridoxine and is presumably able to synthesize the substance. Niacin is probably also to be regarded as a root growth hormone for species in which the roots are unable to synthesize the compound.

To state that thiamine, pyridoxine, or niacin function as root growth hormones does not imply that the same chemical substances may not function as correlative agents in quite different aspects of growth. The evidence available indicates that the growing embryo and its associated tissues such as the endosperm probably also lack the ability to synthesize thiamine but derive this substance by translocation from the leaves. Thiamine is required by the germinating seed and seedling plant, presumably also by the developing embryo. Thus it is entirely likely that thiamine may function not only as a root growth hormone but also as a hormone of seed and embryo development. Neither in the case of the root nor in the case of the seed and embryo is there evidence that the development on the intact plant of the organ in question is directly limited by available thiamine. On the contrary, in most species of plants root growth is controlled primarily by factors other than thiamine. Thiamine, while it is a hormone in the strict sense, is nevertheless not the

TABLE XX

A Summary of the Relationships of Vitamins of the B Complex in a Number of Phases of Plant Growth Development

Vitamin	Physiological behavior						
	Root-growth factor	Leaf-growth factor	Site of synthesis	Translocation in stem	Effect on embryo growth	Synthesis in germinating seed in dark	Other activities
Thiamine	Required by most roots	Inactive	Leaf	Translocated toward roots, growing point, and seeds	Effective on pea	Not synthesized	Required by rose callus in culture
Pyridoxine	Required by some roots	Inactive	Leaf, root in some cases	Translocated toward roots	Ineffective	Probably not synthesized	
Niacin	Required by some roots	Inactive	Leaf? Root in some cases	....	Effective on pea	Synthesized	
Pantothenic acid	Not required	Inactive	Leaf. Root?	Translocated sluggishly	Effective on pea, *Datura*	Synthesized in some cases, not in others	Required by callus of *Crataegus*
Riboflavin	Not required	Inactive	Leaf. Stem? Root	Not translocated	Ineffective	Synthesized	
Biotin	Not required	Inactive	Leaf? Root	....	Effective on pea	Synthesized in some cases, not in others	Cofactor in root initiation in pea
Inositol	Not required	Inactive	....	....	Ineffective	Synthesized	
Adenine and hypoxanthine	Not required	Active on radish pea	Leaf	....	Ineffective except on leaf growth		
Folic acid	Not required	....	Leaf?	....	....	Not synthesized	

key or limiting factor in any developmental reaction of the intact plant which has as yet been studied.

The scattered bits of information brought together in this review indicate that pantothenic acid may, like thiamine, play a hormonal role in seed or embryo development, and the same may possibly be true of biotin. Critical evidence on the translocation of these materials in the plant is needed before a final conclusion can be reached.

It is of interest that in the factors thus far recognized as of importance in regulating growth of the mesophyll of leaves there is no overlapping with factors known to function as growth hormones of root or seed. Concerning the purines adenine and hypoxanthine, it is clear that they are not only active in promoting leaf growth but are also found in organs known to manufacture or store the native leaf growth factors. Critical evidence that the purines are actually the factors translocated in the plant is needed before a hormonal role can conscientiously be ascribed to these substances.

Riboflavin appears to be synthesized in all of the vegetative tissues of the plant and does not, so far as can be ascertained, possess any hormonal role in vegetative growth. Further, it is among the vitamins regularly synthesized in the germinating seed in considerable amounts and consideration must be given to the possibility that it may even be synthesized in the developing seed and embryo. The small amount of evidence available suggests that inositol may constitute a similar case.

The behavior of the several vitamins of the B group in the varied physiological processes which have been discussed are summarized in Table XX. It should be borne in mind, however, that the physiological roles of the B vitamins now recognized may well constitute only a small part of the total number of ways in which these compounds contribute to the integration of plant growth.

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The Influence of the Adrenal Cortex on the Metabolism of Water and Electrolytes

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CONTENTS

I. Introduction

It must be remembered, however, that when nothing was known of the function of the thyroid or of the internal secretion of the pancreas a favorite assumption was that the serious consequences of their removal were due to the loss of a "detoxicating" action, in the absence of which toxic substances which could no longer be neutralized, accumulated in the body. As soon as it was established that thyroid "substitution therapy" was a brilliant success, especially when the specific hormone of the thyroid was isolated, nobody talked any more of the detoxicating function of the gland. It is the same now with the pancreas. It was the same for a time with the adrenals after the discovery of epinephrin. When it was seen, after the first enthusiasm had passed,

that the discovery of epinephrin had not in any important degree solved the riddle of adrenal function, the ghost of the "detoxication" theory began to walk again.—G. N. Stewart (1924).

For several years after Stewart expressed these thoughts in 1924 the ghost of the detoxication theory continued to walk. The observation of Stewart and Rogoff (1925), Rogoff and Stewart (1928) and Corey (1927) that infusion of large volumes of physiologic salt solution or Ringer's solution produced an almost specific effect on the condition of adrenalectomized dogs was first interpreted as a washing out of toxic substances. However, as more detailed chemical studies were carried out it became evident that the distribution and excretion of water and of sodium, potassium and chloride ions were disturbed. The nature of the changes produced by adrenalectomy on the metabolism of sodium, potassium, and chloride and the influence of the adrenal cortex on physiologic processes are more closely examined in the following pages. The adrenal cortex has now joined the thyroid, the pancreas, and the adrenal medulla. The ghost of the detoxication theory walks no more.

II. Survival after Adrenalectomy

The phenomenon that all investigators encountered after adrenalectomy of experimental animals was the development of a condition that Banting and Gairns (1926) characterized for dogs that survived less than 50 hours by rapid pulse, elevated temperature, thirst, loss of appetite, terminal fall of blood sugar, restlessness, suppression of urine, salivation, vomiting, diarrhea, unconsciousness, coma, and convulsions.

The fatal termination in dogs surviving more than 100 hours may begin with suppression of urine, loss of appetite, weakness, especially in the hind legs, drowsiness, and dilatation of the pupils. The superficial veins of the legs collapse and samples of blood are obtained with difficulty. The blood becomes thick and dark. Finally, within a few hours of death there may be vomiting and diarrhea.

Chemical studies on the constituents of the blood after adrenalectomy in the dog revealed a decrease of chloride ion (Lucas, 1926) but Banting and Gairns were not able to prevent death by administration of sodium chloride even when the urea, nonprotein nitrogen, and chloride were maintained at almost normal concentrations.

Marine and Baumann (1927) observed that injections of a solution of sodium chloride produced an increase in the time of survival of adrenalectomized cats, and Baumann and Kurland (1927) added two other essential facts. Associated with the loss of chloride there was a decrease of sodium and an increase of potassium in the serum.

Loeb (1932, 1933) made the outstanding contribution that in patients

who had Addison's disease the concentrations of sodium and chloride in the blood were lower than normal and that there was a striking improvement of the clinical condition when an adequate amount of sodium chloride was given in the diet. A recurrence of the symptoms of severe adrenal insufficiency resulted within 48 hours after sodium chloride was withheld.

Metabolic studies with experimental animals (Loeb *et al.*, 1933) confirmed the observation on the loss of sodium and chloride from the plasma, and it was also found by these same investigators that in dogs the decrease of the content of sodium was more rapid than that of chloride. To control the condition of acidosis which developed because of the greater loss of sodium than chloride Harrop *et al.* (1935) administered sodium bicarbonate with sodium chloride by stomach tube. This treatment maintained adrenalectomized dogs indefinitely without the use of an extract of the adrenal cortex. The concentrations of the constituents of the blood were all within the normal limits except for potassium.

Contemporary with this work it was shown that the high concentration of potassium in the serum could be reduced to normal if the intake of sodium chloride was high and that of potassium was low (Allers, 1935; Allers *et al.*, 1936; Allers and Kendall, 1937). Such a diet was based on the previous work which has been described, and that of Hastings and Compere (1930) which had demonstrated the elevation of potassium in the serum after adrenalectomy.

With the regimen used by Allers development of acidosis was checked with sodium citrate in place of sodium bicarbonate, since the former was better tolerated, and the sodium salts were added to the diet and drinking water. There were two reasons for this method of administration: it involved the least labor, and it seemed desirable to extend the intake of sodium chloride as far as possible throughout the 24 hours of each day. It is difficult to imagine a more simple answer to a complicated and important problem. That the answer was correct has been shown repeatedly.

A word of explanation is necessary to answer some obvious questions. Banting and Gairns (1926), Rogoff and Stewart (1928), Swingle *et al.* (1934a), Zwemer (1934), Loeb *et al.* (1935), and Silvette and Britton (1935) concluded that after adrenalectomy the administration of sodium chloride was helpful but that the survival time was only moderately prolonged. There are many different reasons for this consensus but all of these reasons have one thing in common; that is, they were all based on investigations which failed to satisfy the rather narrow limits of the necessary experimental conditions. Before these conditions had been established there was no known good reason why it was not possible to

furnish food that would be relished and eagerly eaten. With dogs and cats this was meat, and almost invariably in the early stages of the investigation the diets contained a large proportion of this foodstuff (Marine and Baumann, 1927; Loeb *et al.*, 1933; Harrop, 1936a; Harrop *et al.*, 1936). This raised the daily intake of potassium and, as a consequence, the severity of the symptoms was increased, the appetite was impaired, all food except meat was refused, and thus a vicious cycle was produced. It is clear that the eating habits of the animals, the diet, the manner of administration, and the amount and type of sodium salts provided were important factors which shaped the early course of the experimental work.

When it was recognized that the critical condition produced by adrenalectomy was associated with dehydration and an abnormal distribution of water and electrolytes (Loeb *et al.*, 1933) investigations were carried out to determine the extent and cause of these changes. These inquiries, together with other studies of the metabolism of electrolytes, provided quantitative data which are essential for correlation with the functional changes observed.

III. Normal Distribution of Electrolytes

1. Retention of Electrolytes

The intact animal and man can retain sodium, potassium, and chloride over long intervals of time even when the diet contains but small amounts of these ions. This is true whether sodium, potassium, or chloride is individually excluded from the diet or the diet is low in all minerals (Brooke and Smith, 1933; Orent-Keiles *et al.*, 1937; Orent-Keiles and McCollum, 1940, 1941; Greenberg and Cuthbertson, 1942; Cuthbertson and Greenberg, 1945).

2. Stability of Electrolyte Equilibria in Blood and Tissues

Although the diet may be extremely unfavorable, the animal organism is able to maintain this vital balance with little alteration for a relatively long period (Smith and Smith, 1934; Light *et al.*, 1934). A similar resistance to change is shown with a diet which has an unusually high content of sodium chloride and other salts. Marked changes take place only when death approaches and in all probability these changes are responsible for death (Heller and Paul, 1934).

3. Intracellular and Extracellular Compartments

Manery and Hastings (1939) pointed out that muscle cells (that is, the intracellular phase of the tissue) contain a high concentration of

potassium and phosphate, little if any sodium, and no chloride. The remainder, called the extracellular phase, approximates a serum ultrafiltrate in composition and contains all of the chloride and most of the sodium.

As a first approximation Manery and Hastings also concluded (1) that the electrolytes in the extracellular fluid of all tissues are essentially identical, (2) that the mass of the extracellular phase varies from one type of tissue to another, and (3) that tissues have varying proportions of at least two types of cells: those which contain no sodium or chloride, and those which contain sodium and chloride.

The tissues which have a large proportion of chloride-containing cells are blood, connective tissue, gastric mucosa, testes, and probably lung.

Sodium and Chloride. Muntwyler *et al.* (1940b) found the content of sodium and chloride of normal dog muscle to be approximately 28 mM and 20 mM respectively per 100 g. of fat-free tissue.

When radioactive sodium was injected into experimental animals it rapidly permeated all fluids and tissues of the body where sodium is normally present (Manery and Bale, 1941; Greenberg *et al.*, 1940). The results confirmed earlier conclusions that sodium is essentially an extracellular ion. In rats maintained on a low content of potassium but with adequate sodium in the diet the potassium in the muscle was in part replaced by sodium. Radioactive sodium penetrated the muscles of these rats in proportion to the sodium already present (Heppel, 1940). Manery and Bale (1941) and Manery and Haege (1941) found that the radioactive isotopes of sodium and chloride rapidly penetrated the tissue of skin, kidney, liver, muscle, and tendon and were distributed between the tissue and plasma to the same magnitude as the normal isotopes. It was suggested that a comparison of the unstable isotopes of sodium and chloride allows a more reasonable measurement of the extracellular water of many tissues than has hitherto been possible.

Potassium. Miller and Darrow (1940a) found that the potassium in the muscle of rats was usually 47–49 mM/100 g. of fat-free solids but may decrease to as low as 44 mM as a result of a diet with high content of potassium or after an interval of 18 hours following an injection of potassium chloride or of sodium chloride with sodium bicarbonate. Per kilogram of intracellular water, the potassium in the muscle of rats apparently can vary from 146 to 160 mM without a change in the sodium content of the serum. Miller and Darrow further showed that when the potassium in serum is raised by injection of potassium salts the concentration of this cation in the muscle increases quickly to 50 or 53 mM/100 g. of fat-free solids but returns to normal within 90 minutes.

A salt of potassium injected intravenously into dogs was distributed

through a volume of fluid which was much greater than that of the extracellular compartment and corresponded more nearly to that of the total fluid of the body. This indicated that exogenous potassium entered some, and probably most, of the cells of the body (Winkler and Smith, 1938; Houssay and Marenzi, 1937).

The distribution of radioactive potassium confirmed this conclusion (Joseph *et al.*, 1939; Noonan *et al.*, 1941) but indicated further that an excess of potassium in the plasma was probably quickly removed by the viscera and was slowly released to the muscles (Fenn *et al.*, 1941).

4. Functional Changes

Asphyxia, the Tension of Carbon Dioxide, Rate of Blood Flow. Asphyxia for 4 or 5 minutes results in a marked rise of serum potassium. A temporary rise followed breathing a mixture of 10% carbon dioxide and 90% oxygen (Cattell and Civin, 1938), and *in vitro* studies showed that an increase of the tension of carbon dioxide tended to cause potassium to shift from plasma to the muscle (Fenn and Cobb, 1935). Reduction of the rate of flow of blood caused a rapid increase of potassium in the plasma. The source of the potassium appeared to be the muscle or liver (Baetjer, 1935; Mullin *et al.*, 1938; Fenn *et al.*, 1939).

Glucose and Other Metabolites. Potassium leaves the plasma and enters the intracellular compartment after the intravenous injection of glucose, levulose, or pyruvic acid (Flock *et al.*, 1938). A similar change of potassium from solution to cells has been shown for suspended erythrocytes (Danowski, 1941; Harris, 1941) and for yeast in the presence of glucose (Pulver and Verzár, 1940).

Epinephrine. The administration of epinephrine produces a rapid increase of the concentration of potassium in the plasma (D'Silva, 1934; Brewer *et al.*, 1939), and this in turn is followed by a decrease to levels below the normal value (Keys, 1938; Castleden, 1938; Larson, 1940; Stickney, 1941). Perfusion of the liver with a saline solution which contained epinephrine released potassium (D'Silva, 1936).

Contraction of Muscle. When muscle is made to contract by injection of acetylcholine, potassium is liberated (Cicardo and Moglia, 1940a; Somogyi and Verzár, 1941). Contraction of muscle after tetanic stimulation likewise results in release of potassium to the plasma and uptake of water and sodium by the muscle (Fenn and Cobb, 1936; Fenn, 1937; Fenn *et al.*, 1938; Wood *et al.*, 1940; Cicardo and Moglia, 1940b). It is probable that the liberated potassium is taken up in part by the liver. In recovery the reverse changes must occur, since it has been shown that muscle regained the potassium which had been lost. This potassium

cycle is comparable, therefore, to the well-known carbohydrate cycle (Fenn, 1939, 1940).

5. The Distribution of Water

The prevailing opinion as to the behavior of cell fluids was expressed by Peters (1935) as follows: Cells adjust themselves to variations in osmotic pressure of the fluid environment by interchange of fluid rather than of salts. This adjustment would be followed by changes of the cell volume and by variation of the total cellular fluid of the body.

The concentration of hydrogen ion influences the volume of the extracellular and intracellular phases (Hastings and Eichelberger, 1937). The degree of dehydration determines the extent of the changes in the volume of the plasma and of the extracellular and intracellular phases (Wiley and Wiley, 1933). Both the interstitial fluid and the plasma contribute to the fluid lost from the extracellular compartment during dehydration. However, except in the early stages a much larger amount is contributed by the interstitial fluid than by the plasma (Mellors *et al.*, 1942a, 1942b). Ashworth and Gill (1944) showed that changes of fluid in the intracellular compartment followed changes in the osmotic pressure of the extracellular phase in the direction which was predicted.

Darrow (1944) concluded that water, like the other constituents of the body, is in a dynamic state and that all body water is being constantly exchanged. Water is not fixed within extracellular and intracellular phases. The amount of water which is held in a particular location is controlled by many factors. Among these factors is the distribution of sodium and potassium, both of which are also in a dynamic state in which all parts of the body are accessible. A simple type of osmotic relationship does not describe the behavior of body fluids.

IV. Abnormal Distribution of Electrolytes and Water

1. After Adrenalectomy

Sodium, Chloride, and Water. The early investigation of the relation between the adrenal cortex and the loss of electrolytes was confined to a study of changes in the plasma. These results, to which reference has been made, were confirmed by Harrop and Weinstein (1933), Harrop *et al.* (1933a, 1933b), Zwemer and Sullivan (1934), Silvette (1934), Harrison and Darrow (1938), Darrow *et al.* (1939), Muntwyler *et al.* (1940a) and others.

Muntwyler *et al.* (1940b) concluded that the outstanding changes of skeletal muscles of dogs in adrenal insufficiency were a loss of sodium and a gain of water. Associated with these changes was a tendency for the content of chloride to fall. Helve (1940) confirmed these observations.

Since muscle from an adrenalectomized animal contains more water than normal muscle, exposure to Ringer's solution should result in a smaller uptake of water by the former. Evidence for this has been secured by Ponder and Gaunt (1934) and by Angerer and Angerer (1941).

Potassium. Since the first studies on the distribution of electrolytes after adrenalectomy an increase of potassium in the plasma has been observed consistently. The source of the potassium is the diet, for it is possible to maintain the concentration of potassium within normal limits by restriction of the intake (Allers and Kendall, 1937).

Unless the intake of potassium is limited there is an appreciable increase in the concentration of potassium in the muscle when the result is expressed per unit of solids (Hegnauer and Robinson, 1936; Harrison and Darrow, 1938; Muntwyler *et al.*, 1940b). The high values given for potassium in the tissues of the rat by Buell and Turner (1941) after adrenalectomy have been questioned by Darrow (1944).

Other evidence for an increase of potassium in the tissues is given by studies on the metabolic balance of this cation. Both the amount of potassium retained during the interval of adrenal insufficiency and the amount excreted after treatment with cortical hormone are too large to be contained in the extracellular fluids. In the rat the retention of potassium after adrenalectomy can be accounted for by the increased concentration in muscle cells and in the extracellular fluid (Harrison and Darrow, 1938).

2. After Intraperitoneal Injection of Glucose

In 1934–1935 Darrow and Yannet devised an ingenious method to cause a marked loss of sodium and chloride from the extracellular compartment without a significant change of potassium and without loss of body water. This was accomplished by an intraperitoneal injection of an isotonic solution of glucose. Sodium and chloride rapidly diffused into the solution and this resulted in a shift of water to the intracellular phase which caused concentration of the blood and the usual signs of dehydration.

Gilman (1934), through use of this method, showed that the decrease of the volume of the blood even in the intact animal resulted in a condition which in many respects simulated the prostration which follows adrenalectomy. Withdrawal of 6–7 cc. blood/kilogram of body weight reduced the blood pressure to shock level, but after several hours the dog invariably returned to a normal condition. Water again entered the extracellular compartment, the blood became diluted, and there was an increase of the sodium and chloride in the blood to normal values.

When an isotonic solution of glucose was injected intraperitoneally

into adrenalectomized dogs a change of the distribution of water occurred which was the same as observed in normal dogs. However, the effect was much more profound and the experiment was terminated by death. These dogs lacked the power to dilute the blood and redistribute the fluid held within the tissue cells (Parkins *et al.*, 1935; Swingle *et al.*, 1936a; Harrop, 1936b).

Similar observations have been made on cats, rabbits and rats (Robinson and Hegnauer, 1936; Gaunt *et al.*, 1937; Remington, 1940, 1943).

3. After Water Intoxication

The decrease of the concentration of electrolytes in the plasma which follows the intraperitoneal injection of glucose was obtained with only trivial changes of the total amount of water in the experimental animal. Another method to produce a decrease of sodium and chloride in the extracellular compartment is to administer excessive amounts of water. Eventually this brings about a decrease of the osmotic pressure and entrance of water into the intracellular phase. If this hydration proceeds far enough convulsions and death result (Rowntree, 1926).

Swingle *et al.* (1937a) concluded that if intoxication from excessive amounts of water follows hydration of cells, then in such animals the disturbance of the distribution of water and electrolytes is analogous to that found in adrenalectomized animals after the serum electrolytes have been depleted by an intraperitoneal injection of isotonic solution of glucose. Although administration of water to normal dogs decreased the sodium and chloride of the serum, recovery was spontaneous if administration of water was stopped with the onset of convulsions. When healthy vigorous adrenalectomized dogs maintained with cortical extract were treated in the same manner recovery from the prostration and convulsions did not occur.

Protection was afforded normal rats against otherwise lethal water intoxication by the use of an extract of the adrenal cortex and in the adrenalectomized dog or rat the administration of a hypertonic solution of sodium chloride or the hormones of the adrenal cortex increased the resistance of the animal to water intoxication (Swingle *et al.*, 1941; Eversole *et al.*, 1942; Gaunt, 1944a, 1944b; Gaunt *et al.*, 1944; Hays and Mathieson, 1945).

V. Renal Function

A widely accepted concept for the formation of urine by the kidneys is filtration through the glomerulus to yield an ultrafiltrate of plasma

and subsequent reabsorption of electrolytes, glucose, amino acids and other substances together with most of the water in the tubules (Smith, 1937, 1943). The systemic blood pressure is an important factor since the rate of formation of urine, the removal of urea from the blood and filtration through the glomerulus have been shown to vary with the renal blood pressure. The fraction of the plasma which is filtered through the glomerulus depends on the state of constriction or dilatation of the glomerular arterioles, on the permeability of the glomerular capillaries, on the intrarenal or capsular pressure, and perhaps on the period of contact of the blood with the glomerular capillaries (Smith, 1937, 1943).

Tubular reabsorption of urea and electrolytes is modified by the rate of flow of urine, since in case of low rate more time is available for return of solutes from the urine into the blood stream, whether by diffusion (Holten and Rehberg, 1931) or by other mechanism (Shannon, 1936). In addition, there are many examples of a delicately balanced mechanism which is not well defined. The excretion of one substance may influence the reabsorption of another by the tubules. The infusion of hypertonic solutions of sodium and potassium chloride suppressed the reabsorption of ascorbic acid (Selkurt and Houck, 1944).

The excretion of sulfate is somewhat increased by the simultaneous injection of sodium chloride, but excretion of chloride is almost completely repressed by the sulfate ion, even when a large infusion of sodium chloride is given simultaneously (Schwartz *et al.*, 1942).

VI. Renal Function after Adrenalectomy

1. The Excretion of Sodium and Chloride

Loeb *et al.* (1935) assembled the available data and concluded that the decrease of the concentration of sodium and chloride in the blood which occurs in Addison's disease and in adrenalectomized animals was because of loss of these electrolytes through renal excretion. Somewhat earlier Loeb *et al.* (1933) had concluded: "From the evidence presented it is difficult to ignore the possibility that this assumed influence of the adrenal glands may be exerted on renal function." Harrop *et al.* (1933a) expressed the belief that one function of the cortical extract in the adrenalectomized dog is that of participation in the regulation of the metabolism of sodium and chloride: "The available evidence points to the kidney as the locus of this regulatory function of the cortical hormone."

The changes which occur in different species of experimental animals are not the same. During adrenal insufficiency in man, the cat, and the dog, sodium and chloride are rapidly lost through the kidney

(Harrison and Darrow, 1938), but in the opossum this is not so (Britton and Silvette, 1937a; Hartman *et al.*, 1943). The concentration of sodium and chloride in the plasma may increase (Silvette and Britton, 1936). The response of the adrenalectomized opossum to the ingestion of a solution of sodium chloride and of the normal opossum to an extract of the adrenal cortex was less than that shown by other mammals (Silvette and Britton, 1938; Smith *et al.* 1943).

Sandberg *et al.* (1937) did not find an increase of the concentration of sodium in the urine of the adrenalectomized rat. The concentrating power of the kidney was impaired but in the adrenalectomized dog both the total amount of sodium and its concentration in the urine were increased.

2. The Excretion of Potassium

Harrison and Darrow (1939) found that after adrenalectomy, associated with the loss of sodium, the concentration of potassium in the urine was much lower than that found in normal animals with comparable concentrations of these ions in the plasma. The limiting ratio of the potassium in the urine to that in the plasma (the U/P ratio) was approximately 10:1 compared to 40:1 to 75:1 for the normal dog. Keith and Binger (1935) have shown that in man under normal conditions the concentration of potassium in the urine may be 50 times that in the plasma. Harrison and Darrow (1939) concluded that the abnormality of the excretion of potassium after adrenalectomy was not dependent on the change in the glomerular filtration, and failure to concentrate potassium in the urine was not because of inability of the kidney to do osmotic work nor to diuresis. The total excretion of electrolyte was not low, and even at the same volume of urine the administration of an extract of the adrenal cortex rapidly increased the U/P ratio for potassium. The disturbance of function involved the differential absorption of potassium by the tubules of the kidney.

3. Glomerular and Tubular Activity

In the untreated adrenalectomized dog it appears probable that both the rate of glomerular filtration and that function of the renal tubules which is concerned with the differential reabsorption of electrolytes are impaired (Gersh and Grollman, 1939). Harrison and Darrow (1939) suggested that as a result of the disturbed function of the tubules the concentrations of electrolytes in the body fluids are not maintained at normal levels. The decrease of electrolyte is followed by a shift of water from the extracellular to intracellular spaces and a decrease of the volume of the extracellular fluid. As a consequence of this the volume of the

plasma is decreased and in turn the blood pressure is diminished. In the adrenalectomized dog, and in the intact dog depleted of sodium and chloride, the decreased rate of glomerular filtration, which may be only 25% of the normal value, can be explained by the decrease of blood pressure. As the terminal stage of adrenal insufficiency approaches, the decrease of blood pressure would be still further aggravated by other factors such as a change of the muscular tone of the vascular system. Harrison and Darrow (1939) have postulated that the retention of urea in adrenal insufficiency can be adequately explained by the decreased rate of glomerular filtration together with an increased reabsorption of urea which is secondary to the reduced glomerular filtration.

These same investigators also pointed out that in the intact animal during the process of differential reabsorption of water and electrolytes in the renal tubules high concentration gradients between tubular urine and blood plasma may be developed. After adrenalectomy in the dog there is a marked disturbance of renal function of a specific type. Sodium is not adequately reabsorbed from the glomerular filtrate at a time when the concentration of sodium in the plasma is low. Potassium is reabsorbed to a much greater extent in the renal tubules of the adrenalectomized dog than in the normal animal at a time when the concentration of potassium in the plasma is abnormally high.

4. The Excretion of Water

Moderate diuresis frequently accompanies the increased excretion of sodium and chloride after adrenalectomy or after cessation of treatment of an adrenalectomized dog with cortical extract (Banting and Gairns, 1926; Corey, 1927; Loeb *et al.*, 1933; Swingle *et al.*, 1934c; Harrop, 1936b). However, the increased loss of water through the kidneys is but transitory and after a few days if water is administered diuresis does not follow (Gaunt, 1944a). The abnormality which develops in adrenal insufficiency may be influenced by four possible causes: (1) delayed absorption of water from the gastrointestinal tract; (2) an abnormal amount of the antidiuretic hormone in the blood; (3) an abnormal distribution of water in the body; (4) faulty excretion by the kidney (Gaunt, 1944b; Levy *et al.*, 1946).

1. The absorption of water from the gastrointestinal tract is delayed but this cannot be the only or the most important cause for the failure to produce diuresis.

2. Both the adrenal cortex and the antidiuretic hormone are concerned with water metabolism. Adrenalectomy in cats appears to be followed by the excretion of increased amounts of an antidiuretic substance (Martin *et al.*, 1939). The results of Joseph *et al.* (1944) on

adrenalectomized rats indicated almost complete repair of the delayed diuresis by small doses of cortical extract. Gaunt (1944a) concluded that the posterior lobe of the hypophysis can be eliminated by implication from the responsibility for the antidiuretic influence found in adrenalectomized rats, for hypophysectomized rats failed to show a normal diuretic response to water and Chen and Geiling (1943) reported that this occurred whether the posterior lobe was intact or removed. Since it was found that the failure to produce diuresis in the hypophysectomized rats was relieved by administration of cortical hormones it follows that the primary fault was adrenal insufficiency. By implication the posterior lobe of the hypophysis was not of first importance as a cause for failure to show a normal diuretic response to water after adrenalectomy.

3. Some degree of hydration of the intracellular compartment is usually a sequel to insufficiency of the hormones of the adrenal cortex. Although concentration of the circulating fluid is encountered only in severe insufficiency the tendency for hydration of the cells is apparent at an early stage. This situation would probably modify the availability of water for excretion by the kidney.

4. To explain the failure of the normal diuretic response to water as faulty excretion by the kidney, only little help, if any, is given by a histologic study of the kidney. The appearance of the structure is usually essentially normal (Levy *et al.*, 1946).

It is probable that all four causes which have been discussed play a part. Shipley (1945) concluded that although there is a demonstrable delay of the intestinal absorption of water in adrenalectomized rats and a decreased emptying time of the stomach, these are only minor causes of failure of excretion when water is ingested in large quantities. The greatest portion of the administered water is retained in the extracellular compartment and probably remains unexcreted because of failure of elimination by the kidney.

VII. The Transfer of Water and Electrolytes through the Intestinal Wall

Visscher and his associates have proposed a hypothesis for the transfer of water and certain electrolytes between the lumen of the intestine and the blood, which indicates a possible explanation for some of the observations recorded in the preceding pages. They pointed out that the ability of living systems to perform osmotic work is generally recognized but that the mechanism by which such processes occur has not been elucidated. The renal tubules are able to bring about the movement of chloride from the glomerular filtrate to the plasma until the concentration of chloride within the tubules is virtually zero. To move the last portions

of the chloride the renal cells transport ions from one concentration to another which is at least 100 times as great. Such a process as in the kidney is difficult of analysis, for it is necessary to deal with microscopic structures and very small amounts of material. It was shown that a comparable phenomenon could be made to occur in the small intestine of the dog and there was thus made available a convenient preparation for the study of the transportation of ions (Ingraham and Visscher, 1936a).

In summary, the mechanism suggested is a simultaneous forced flow of water across the intestinal epithelium in both directions. Differences of the electrolyte content of the water in the two streams and the relative rates of the streams determine the direction and magnitude of the net transport (Visscher *et al.* 1944b).

This conclusion was formed through the use of deuterium oxide and isotopic sodium and chloride. The experimental results were found to differ as much as 200-fold from the values which would be obtained if the movement of water, sodium, and chloride was by diffusion. In certain instances the direction of movement was the reverse of the predicted. The observations refute the commonly held belief that net water movement between the ileum and the blood occurs primarily because of normal osmosis. They are also incompatible with the view that differences of concentration in the normal intestine and in the blood are the major causes for the movement of sodium and chloride ion between the two fluids.

The apparent concentrations of the sodium and chloride ions in the water which passed from the lumen of the intestine into the blood were proportional but not always equal to the concentrations of these ions in the intestine, but the apparent concentrations of sodium and chloride in the water which moved from the blood into the intestine were practically independent of the concentrations of these ions in the intestine. The turnover of sodium between the intestine and the blood appears to be rapid and the results indicate that an amount of sodium approximately equal to the total sodium in the plasma may be replaced in less than 100 minutes (Visscher *et al.* 1944a).

When distilled water or hypotonic solutions of sodium chloride or of urea were placed in the small bowel of a dog the concentrations of sodium, chloride, and urea increased to values which were greater than their respective concentrations in the blood. However, in the presence of the sulfate ion, chloride did not accumulate but on the contrary was removed from the bowel until its concentration was much less than that of the blood plasma.

The sulfocyanate ion in the bowel affected the movement of chloride through the intestinal wall in the opposite way from that of sulfate and

caused the chloride ion to accumulate to a concentration which was greater than that in the blood plasma (Burns and Visscher, 1935).

The poisons, arsenite, fluoride, sulfide, cyanide, and mercuric chloride, abolished the action of sodium sulfate and in the presence of these poisons chloride moved into the intestine under circumstances such that it would otherwise move out. In addition, in the presence of these poisons the intestinal wall, which normally was impermeable to sulfate, became permeable to it (Ingraham and Visscher, 1936b).

The results of Peters (1941) supported the experimental work on the circuit hypothesis and Driver (1942) also found that in the presence of sulfate ion, chloride rapidly passed from the lumen of the intestine. Hexylresorcinol and other agents were shown to modify the transport of electrolytes through the intestinal wall.

At the present time investigations to clarify the factors which influence the transfer of electrolytes have been limited, but Dennis and Wood (1940) have shown that after adrenalectomy there are changes in the absorption of ions from the intestine similar to those observed by Harrison and Darrow (1939) in the kidney. There was a tendency for the absorption of potassium from the lumen of the intestine and for loss of sodium from the blood into the intestine. Whether these changes were brought about by a specific effect of the state of adrenal insufficiency has not been shown; however, administration of an extract of the adrenal cortex corrected this abnormal behavior.

VIII. The Relation between Sodium Salts and the Hormones of the Adrenal Cortex

Perhaps the most significant single fact which has resulted from many years of investigation of adrenal insufficiency is the specific nature of the effect of sodium chloride. Completely adrenalectomized dogs which have been maintained for months with the optimal amounts of sodium, potassium, and chloride are alert, vigorous, and strong and to all outward appearance they cannot be distinguished from normal dogs. Furthermore, the mineral and organic constituents of the blood and tissues are normal and histologic examination of the muscles, the gastrointestinal tract, the kidneys, the glands of internal secretion, and other organs reveals the fact that except for enlargement of the thymus and the lymph nodes these structures are essentially normal.

These results would indicate that loss of sodium chloride through the kidney is a principal cause for the severe prostration, asthenia, and death after adrenalectomy and that restoration of a normal condition is dependent on replacement of the lost electrolytes. As a general description of the course of events this statement is correct, but when a detailed

study is made it becomes clear that the relationship between many physiologic processes and the intake of sodium chloride is complex. The effects are so broad in scope that it is impossible to select any primary locus. The following paragraphs record some of the relationships that have been observed.

IX. The Influence of Sodium Chloride on Physiologic Processes

1. *Renal Functions*

Water. The influence of sodium chloride on renal function was shown by the response of adrenalectomized dogs to intravenous injection of isotonic solutions of glucose. If 0.9% sodium chloride was present in the solution adrenalectomized dogs responded like normal dogs. Diuresis was induced but the glucose was not injected at a rate fast enough to cause glycosuria, and there were only minor changes in the electrolytes of the blood after the solution had been injected for 3 hours. It was also found that an equally satisfactory result was obtained if the sodium chloride was omitted, but an extract of the adrenal cortex was given. However, if the isotonic solution of glucose alone was injected the dogs became anuric and severe symptoms of adrenal insufficiency developed (Kendall *et al.*, 1938). Gaunt (1944a) found that the injection of only 1 cc./hour of physiologic salt solution into normal rats while large doses of water were being given by mouth increased diuresis, extended life, and prevented the rise in the percentage of cells in the blood as measured by the hematocrit.

Sodium and Potassium. Anderson (1943) suggested that the administration of sodium chloride not only replenished the body with the needed ions but also restored to the kidney the "mechanism which is responsible" for the normal excretion of sodium and potassium. To demonstrate this, radioactive sodium and potassium were given to rats at various times after adrenalectomy and it was shown that after 8 days an average of 60% of the ingested radioactive sodium was excreted during a 48-hour interval when the rats drank tap water. The average amount excreted by normal rats was 37% and the value for adrenalectomized rats maintained with 1% solution of sodium chloride was 36%. For potassium the value for the normal animal was 10%. Adrenalectomized rats given tap water for 8 days excreted only 5%; however, when adrenalectomized rats were given 1% solution of sodium chloride for intervals of 5 and 8 days, 10 and 11% respectively of the radioactive potassium was in the urine.

At the present time it is difficult to interpret this apparent return of normal renal function in any greater detail than Anderson's suggestion

that the mechanism responsible for the normal excretion of sodium and potassium had been restored by the ingestion of sodium chloride.

Kottke *et al.* (1942) found that after the adrenalectomized dog had been maintained in good condition for an interval on a diet with high sodium and low potassium content the capacity of the kidney to excrete a dilute urine was only slightly, if at all, impaired when compared with the capacity of the kidney of the intact animal. The kidney of the adrenalectomized dog was not able to produce as concentrated a urine nor one with as high a content of sodium chloride as the kidney of an intact dog. However, to show these slight limitations it was necessary to resort to severe measures during which the temperature of the environment was elevated.

These results, which are in keeping with those of Anderson, show how nearly normal the kidney had been maintained without the hormones of the adrenal cortex by the use of a diet which had an optimal concentration of electrolytes.

Excretion of Urea. Banting and Gairns (1926) appear to have been among the first investigators to demonstrate the close relation between the excretion of urea and the administration of sodium chloride in the adrenalectomized dog. They reported that in the morning of a certain day the concentration of urea of an adrenalectomized dog was 315 mg./100 cc. blood. In the afternoon, despite the fact that the dog had received 300 cc. of serum, the concentration of urea was 353 mg. Two hundred cubic centimeters of 5% saline solution were given and on the following morning the blood urea had decreased to 109 mg. Two hundred and seventy-five cubic centimeters of 5% saline solution given during the day reduced the concentration of urea to 90 mg. in the afternoon and the following morning the value was 60 mg.

This work had been preceded by that of Marshall and Davis (1916) who showed a marked rise in the blood urea after adrenalectomy, and it was followed by the observations in many laboratories that an increase of the nonprotein nitrogen and urea in the blood are invariable sequelae to adrenalectomy.

Harrison and Darrow (1939) have pointed out that there are adequate reasons for the increase of urea, which are based on the abnormal distribution of water in the intracellular phase, the extracellular phase and the volume of the blood; and these investigators showed that a decrease of the content of sodium chloride in the blood, produced by an intraperitoneal injection of glucose, will result in an almost complete suppression of the excretion of urea.

Swingle and Remington (1944) suggested several reasons for a decrease of the clearance of urea, among which is a decrease of blood pressure. Swingle *et al.* (1934b) observed that the increase of the con-

centration of urea in the blood occurred simultaneously with the fall of the arterial pressure and did not precede it. It was believed that failure to excrete urea was probably extrarenal in origin and was not necessarily related to the kidney. However, the phenomenon appeared to be related to the change of the concentrations of the electrolytes in the blood.

Adrenalectomized dogs, when given small sustaining amounts of cortical extract and fed ground meat which had been freed of potassium by boiling in water, may be strong, vigorous, and with good appetite with a concentration of urea of from 100 to 400 mg./100 cc. blood. These experiments were not acute nor of short duration. The concentration of urea could be held at almost any desired value by variations of the daily intake of sodium chloride (Kendall, unpublished results). These results may be explained quite simply on the grounds already suggested (that is, a decrease of blood pressure or a decrease of the extracellular compartment), but it is possible that something more specific for renal function is supplied by sodium chloride. As with the excretion of water and of sodium and potassium ions, sodium chloride may in part "restore the mechanism responsible" for the excretion of urea by the kidney.

2. Intestinal Absorption

Electrolytes. Dennis and Wood (1940) found that administration of sodium chloride to an adrenalectomized dog restored the function of the intestine to such a degree that the constituents of the blood were essentially normal and the dog was objectively in excellent health; however, the behavior of the intestine was not normal. The rate of absorption of sodium in general declined more than that of potassium and there was often a reversal in direction of the net movement of sodium, which was sometimes excreted into the lumen of the intestine although potassium was still being absorbed.

Clark (1939) and Stein and Wertheimer (1941), with adrenalectomized rats maintained with sodium chloride, fully confirmed the results of Dennis and Wood. It was also shown that resorption of sodium chloride from a low concentration in the intestine when the concentration in the blood was higher was no longer possible.

Glucose. Verzár (1939) contended that there is a failure of the selective absorption of metabolizable sugars after adrenalectomy. Clark and MacKay (1942) have adequately explained this observation. They found that the rate of absorption of glucose was markedly reduced when measured immediately (24 hours) after the removal of the adrenal glands. When adrenalectomized rats were given sodium salts and maintained for 2 weeks after operation but were not allowed salt water during a period of 36 hours before testing, or if only tap water was given throughout the

period of observation, the absorption of glucose was significantly lower than in the controls. When administration of sodium chloride was continued until the observations on the absorption of glucose were made, the rate of absorption in the adrenalectomized rats was the same as that in the controls. It was concluded that the impaired absorption of glucose evident in adrenalectomized animals under some conditions was not referable to the lack of the influence of the hormones of the adrenal cortex on the intestinal mucosa, but rather to the consequent disturbance of salt metabolism. Similar results have been reported by Anderson (1943) and by other investigators cited in the work of Clark and MacKay (1942).

Fat. Laszt and Verzár (1936) found that after adrenalectomy in the rat absorption of fat was decreased. This result was interpreted as a failure of phosphorylation of the fat in the absence of the adrenal cortex, but Barnes *et al.* (1941a) reported that when the adrenalectomized rats were maintained in good condition with sodium chloride the absorption of fat was normal. Subsequently this conclusion was modified (Bavetta *et al.*, 1941; Bavetta, 1943; Bavetta and Deuel, 1942) to the extent that the lower fatty acids up to caproic are absorbed at a normal rate by the adrenalectomized rat maintained with sodium salts. However, under the same experimental conditions the higher fatty acids are not removed as rapidly from the intestine of the adrenalectomized rat as from the intestine of the normal rat. In adrenalectomized animals maintained on water alone, Bavetta and Deuel (1942) showed that the absorption of sodium butyrate was significantly lowered but the rate was restored to normal by administration of sodium chloride. It was suggested that the beneficial effect of sodium salts was through dilution of the blood and restoration of a more normal circulation. When excretion of fat and fatty acids in the feces was used as a criterion it was found that adrenalectomy did not influence the absorption of fat in rats, provided the intake of food was adequate and the rats were maintained with sodium chloride in the drinking water (Clark and Wick, 1939).

3. *Metabolism of Protein*

Anderson (1943) and her associates have investigated the influence of adrenalectomy on growth, consumption of food, and the need for the optimal intake of sodium chloride. Without the addition of sodium chloride growth stopped, and the food and water intake decreased. With a daily intake of 700–900 mg. sodium chloride the rats survived indefinitely and the rate of growth was almost the same as that of normal controls. An intake of about 350 mg. was insufficient for growth or survival and when the intake was approximately 1,200 mg. the rats

resembled those that were untreated; the survival time was less than 20 days.

The adrenalectomized rat with optimal amounts of sodium chloride is apparently able to utilize exogenous protein almost as well as is the normal rat. After treatment with phlorhizin, the adrenalectomized rat is fully able to convert exogenous protein into glucose (Wells and Kendall, 1940b), but Evans (1936), Long *et al.* (1940) and Anderson (1943) have shown that the adrenalectomized rat cannot readily utilize endogenous protein during a fast, and a limited ability of the adrenalectomized rat after treatment with phlorhizin to convert endogenous protein into glucose was found by Wells and Kendall (1940b).

Under fasting conditions, when the optimal intake of sodium chloride was given, not only were adrenalectomized rats able to survive longer than when the intake of sodium chloride was not optimal but the excretion of more nitrogen indicated that the rats were able to utilize endogenous protein to a greater extent. With too little or too great an intake of sodium chloride the utilization of protein was decreased (Anderson, 1943).

More recently Ingle and Oberle (1946) have completed work which increases the significance of sodium chloride in relation to the metabolism of endogenous protein. Rats were first adapted for a period of 2 weeks to a regimen of forced feeding and were then adrenalectomized. The adrenalectomized rats were given a 1% solution of sodium chloride to drink during all phases of the experiment. During the first postoperative day the adrenalectomized rats excreted less nitrogen than before adrenalectomy, but by the third day there was an increase of the excretion of nitrogen. The higher rate of excretion was sustained for several days and was greater in the adrenalectomized rats than in the control intact rats. During a 10-day fast the adrenalectomized rats excreted as much nitrogen as did the control animals.

These results, and also those of Anderson, furnish positive evidence for the ability of an adrenalectomized rat to utilize endogenous protein. Groat (1941) reported that fasted adrenalectomized ground squirrels given 1% solution of sodium chloride to drink did not survive any longer than did a similar group given tap water. Groat also reported that adrenalectomized rats given 1% solution of sodium chloride to drink did not survive longer than adrenalectomized rats given tap water when the rats were fasted or given a limited amount of food.

It is possible that these results may be explained by failure of the adrenalectomized animals to become adapted to the conditions imposed by the fast, but whatever explanation is given the result is negative in nature and is not confirmed by subsequent work. This is mentioned

because Darrow (1944) referred to the work of Groat and drew the conclusion that "these experiments give further evidence that adrenal insufficiency is not simply a matter of water and electrolyte balance."

The work of Ingle and Oberle (1946) indicates that in rats that have been adapted to the experimental conditions, as long as there is a balance of water and electrolytes, those processes concerned with utilization of endogenous protein proceed as well in the adrenalectomized rat as in the normal rat. Moreover, during a fast a severe strain is imposed on the animal, not only in regard to the utilization of endogenous protein but in the maintenance of homeostasis under adverse conditions. Even this test has now been passed satisfactorily by the adrenalectomized rat when maintained with the optimal intake of electrolytes.

4. Metabolism of Carbohydrate

Deposition of Glycogen in the Liver. Long *et. al.* (1940) have reviewed the influence of the adrenal cortex on carbohydrate metabolism. It has been a subject of much investigation and it has also been a source of controversy since 1930, when Britton and his associates attempted to relate the function of the adrenal cortex to the observation that in the adrenalectomized animal under certain conditions only small amounts of glycogen were present in the liver and muscles, and that hypoglycemia was an almost constant sequel to adrenal insufficiency. Throughout the following decade the dependence of carbohydrate metabolism on the hormones of the adrenal cortex and the secondary importance of "the water and salt hormone" were emphasized by Britton and his associates (Britton and Silvette, 1931, 1932a, 1932b, 1934, 1937b, 1937c; Britton, 1932; Silvette and Britton, 1932, 1936; Britton *et al.*, 1938).

While the close link between the hormones of the adrenal cortex and carbohydrate metabolism was being emphasized by Britton and his associates, results in other laboratories tended to minimize this phase of adrenal insufficiency. The conclusion was drawn that after adrenalectomy carbohydrate metabolism was not primarily disturbed (Parkins *et al.*, 1936). Deuel *et al.* (1937) found that the deposition of glycogen was not altered by adrenal insufficiency if the rats were maintained in good condition by oral administration of sodium chloride. However, two years later Britton (1939) reported that the adrenalectomized cat maintained with solutions of glucose and sodium chloride by mouth was unable to fix or synthesize glycogen in the liver.

Long *et al.* (1940) bridged the gap with a probable explanation for the divergence in experimental results. They pointed out that "the conditions under which a solution of this problem may be attempted require the recognition of certain complicating factors that are encountered in

dealing with adrenalectomized animals. The first of these is the question of the food ingested prior to the glycogen analyses. In some species, particularly the cat, all food may be refused for several days before death, while in others food may be eaten up to a few hours before it occurs. Obviously, this difference will greatly influence the levels of carbohydrate in the tissues when the examination is made. Arising from this is a second problem. Britton and his colleagues have recently reaffirmed that not only do adrenalectomized animals of several species exhibit reduced carbohydrate levels, but they also show a much decreased ability to form liver and muscle glycogen from ingested carbohydrate." Long *et al.* (1940), in agreement with Deuel *et al.* (1937), showed that adrenalectomized rats or mice, maintained in good health by the oral or intraperitoneal administration of sodium salts and fed glucose, showed no marked abnormalities in the storage of carbohydrate. However, when fasted, there was a rapid decline of the hepatic glycogen and an abnormal fall of muscle glycogen which became progressively greater as the fast was continued.

Anderson (1943) and her associates added the important point that the maximal deposition of glycogen in the liver occurred only with the optimal intake of sodium chloride. The deposition of glycogen after absorption of fed glucose in adrenalectomized rats was close to the amount found in normal rats when both groups were given the optimal amount of salt. This was 1% of sodium chloride in the drinking water; however, if the intake of salt was too low or was more than optimal, then less glycogen was deposited in the liver. Furthermore, the beneficial effect of sodium chloride on the deposition of glycogen was not temporary in its action. The capacity of the adrenalectomized rat in this respect persisted for several months if the rat was maintained with 1% sodium chloride in the drinking water.

At the end of the decade it was shown that the secretion of the adrenal cortex contains not one but a group of hormones. Some exert an effect on the storage of carbohydrate and these have but a limited influence on the metabolism of electrolytes. Others have a specific effect on the excretion and distribution of electrolytes, but these do not modify in a significant way the storage of carbohydrate. Long *et al.* (1940) furnished convincing evidence for the effect of corticosterone, dehydrocorticosterone, and the 17-hydroxyl derivatives of these two hormones on the deposition of glycogen in the liver.

Gluconeogenesis. In addition to the deposition of glycogen in the liver of adrenalectomized animals after the ingestion of foodstuff, it was reported by Britton and Silvette (1932a, 1932b) that the injection of an extract of the adrenal cortex increased the blood sugar and the glycogen

in the muscle. These observations were confirmed by Thaddea (1935) but Parkins *et al.* (1936) failed to note a significant effect of the extract of the adrenal cortex on blood sugar. At the time when these experiments were performed there was always a question whether traces of epinephrine or other toxic compounds were present in the extracts of the adrenal cortex that were used, so that it was difficult to evaluate the results.

When corticosterone and its derivatives became available it was possible to study the effects of these pure crystalline hormones on carbohydrate metabolism. This study was carried out by Long and his associates in a convincing manner (Long *et al.*, 1940). They were able to show that certain crystalline hormones of the adrenal cortex increased the level of glucose in the blood of both normal and adrenalectomized rats and mice. In addition the total store of carbohydrate in the body was increased and this occurred even when the animals were fasted before the hormones were administered.

This effect on carbohydrate metabolism was apparently confined to corticosterone, dehydrocorticosterone, and the two derivatives of these compounds with a hydroxyl group at C_{17}.

The increase of the concentration of blood sugar confirmed the results obtained by Britton and his associates but the rise in the glycogen of the muscle which had been reported by Britton was not confirmed. The increase which was found by Britton was ascribed by Long *et al.* (1940) to the manner in which the experiment was performed.

When it was evident that the total carbohydrate stores in the body were increased by treatment with the hormones of the adrenal cortex it became a matter of importance to explain the source of the newly formed sugar. It was suggested by Long *et al.* (1940) that, since there was an increase of the excretion of nitrogen, and in the absence of experimental evidence for the transformation of fat to carbohydrate, the most probable source seemed to be protein. The increase of the excretion of nitrogen indicated the utilization of an amount of protein which could account for the gluconeogenesis.

In the experiments that have been described the adrenalectomized rats were maintained in good health with a high daily intake of sodium chloride. In this condition, even during a fast, the conversion of protein to glucose proceeds at a rate sufficient to sustain the concentration of glucose above the level at which symptoms of hypoglycemia appear; however, through the use of sodium chloride alone the rate of utilization of endogenous protein cannot be increased to the point at which glycogen is deposited in the liver. Evans (1936), and Wells and Kendall (1940b) have shown that even the stimulus to protein catabolism which is asso-

ciated with phlorhizination does not result in a high excretion of glucose by the adrenalectomized rat when maintained on sodium chloride alone. The administration of corticosterone and related hormones increased the glycosuria to that observed in the normal rat. The source of the glucose in this case was apparently protein since the D:N ratio was 3.7:1.

5. Metabolism of Fat

Verzár and Laszt (1936) noted that the fatty livers normally found in rats that have been poisoned with phosphorus did not develop if the animals were adrenalectomized. In such animals administration of an extract of the adrenal cortex was followed by deposition of fat in the liver. MacKay and Barnes (1937) and MacKay (1937) investigated the deposition of fat in the liver in a wide range of conditions such as during fasting, after the existence of a state of alkalosis, or if the animal was given heavy cream or a high fat-low protein diet.

Since the deposition of fat in the liver under these conditions did not occur in the adrenalectomized rat although under the same conditions fat was deposited in the liver of the intact animal, MacKay and associates became interested in the influence of adrenalectomy on fat which had previously been stored in the liver. It was found that in the normal animal the excess fat gradually left the liver over a period of several days, but if the rats were adrenalectomized when the fast was begun the fat stored in the liver was removed from this organ much more rapidly.

The prevention of fatty livers in adrenalectomized rats was observed even though they were maintained in good condition with sodium chloride, but if the rats were given an active extract of the adrenal cortex they behaved as normal rats.

Barnes *et al.* (1941b) confirmed the observation that fat was not deposited in the liver in adrenalectomized rats maintained with sodium chloride. Fatty acids with a strong absorption of radiation in the ultraviolet region through the effect of conjugated double bonds were used to follow the rate of phosphorylation and deposition in the liver. The results indicated that in the adrenalectomized rat maintained with salt the tagged fatty acids were not deposited in the neutral fat of the liver within 8 hours after the fat was fed, but when cortical extract was given, the tagged fatty acids were deposited as neutral fat in the liver.

Observations of the incorporation of tagged fatty acids into the phospholipid fraction of the liver indicated that in the adrenalectomized rats maintained only with sodium chloride there was a normal or possibly an increased rate for the phosphorylation of the fatty acid. Treatment with cortical extract did not modify this phase of the metabolism of fat.

6. Metabolism of Potassium

There are strong indications that one of the most important reasons for the favorable effect of sodium chloride in adrenal insufficiency is the specific influence exerted by this salt on the distribution and excretion of potassium.

When adrenalectomized dogs were maintained with a high intake of sodium chloride and sodium bicarbonate an inverse relationship was found between the concentration of sodium and potassium in the serum (Harrop, 1936a). If the concentration of sodium was below the normal value because of an inadequate intake then the concentration of potassium increased, but if the intake of sodium salts was sufficient the abnormally high level of potassium decreased, often to the normal value. This decrease of potassium in the serum was associated with an increase of its urinary excretion, frequently with a well-marked diuresis.

Nilson (1937) showed that the asthenia and prostration which are induced by the ingestion of potassium salts by an adrenalectomized dog can be relieved by the administration of sodium chloride and sodium citrate alone. If the condition of the animal was not critical it was not necessary to resort to the use of an extract of the adrenal cortex to restore the normal condition. The beneficial effect of sodium salts in Nilson's experiment was closely associated with the decrease in the concentration of potassium in the serum.

Harrison and Darrow (1938) stated that "the mechanism by which changes in the concentration of sodium in extracellular fluid may influence the concentration of potassium within the muscle cell is unknown." Buell and Turner (1941) expressed a similar thought: "The mode of action of sodium chloride in compensating for the specific effects of adrenal insufficiency in the muscles is not clear. There is no evidence here of increased permeability of cell membranes; in fact, there appears to be less sodium rather than more inside the muscle cells. The fact remains, however, that treatment with sodium chloride has in some way prevented the increase in muscle potassium which would have occurred in its absence."

The demonstration that there is not only an inverse relation between the intake of sodium chloride and the excretion of potassium but that an inverse relation also applies to the concentrations of sodium and potassium in the plasma, together with the observations of Miller and Darrow that the concentration of potassium in the muscle varies with that in the plasma, provides the necessary conditions for the specific effects of sodium chloride. However, the details of the mechanism which is the basis for the inverse relationship between the electrolytes have not been revealed.

It has been shown by Heppel (1939) that the influence of sodium chloride on the metabolism of potassium reaches still further. Even in normal rats a large fraction of the potassium within the muscle cells can be replaced with sodium. This was accomplished by use of a diet in which the content of sodium chloride was high but that of potassium was low.

Following the work of Heppel (1939) on replacement of potassium in muscle with sodium *in vivo*, Steinbach (1940) showed that as potassium was soaked out of frog muscle by treatment with potassium-free Ringer's solution, sodium entered in exchange. The exchange was reversible as long as the muscles did not lose more than about half of the original potassium, and provided that the chloride content of the tissue remained about the same.

7. *Miscellaneous Effects*

Appetite. A clear demonstration of the general systemic effect of sodium chloride on adrenalectomized rats is furnished by the procedure devised by Groat (1941). Adrenalectomized rats were maintained with 1% solution of sodium chloride. The salt solution was then replaced with water. When it appeared that the rat would succumb from adrenal insufficiency the water was replaced with 1% solution of sodium chloride. Substitution of water for the salt solution was followed by a loss of weight. After three days the average body weight was approximately 90% of the starting weight and the average intake of food was only 45% of the former intake. Even the most extreme of 3-day fluctuations in the food intake of normal rats as found by Groat were small in comparison to these figures. After replacement of the water with 1% solution of sodium chloride it was observed that the rats immediately began to drink. A large quantity of the solution was consumed within an hour or two and the rats then exhibited a normal appetite. In a group of six animals the average gain in weight after 1 hour was about 25 g., most of which was water.

Some interesting observations (Richter, 1936b, 1941; Richter and Eckert, 1938; Clark and Clausen, 1943) indicated the apparent wisdom of adrenalectomized rats when given a choice of food and water with and without added sodium chloride. The rats voluntarily selected a diet which afforded a high daily intake of salt. The probable basis for this selection was an increase in the sensitivity of the sense of taste to salt (Richter, 1939).

Spontaneous Activity and Response to Stimulation of Muscle. Richter (1936a) observed that the spontaneous running activity of rats rapidly decreased to zero after adrenalectomy and that when small amounts of sodium chloride were added to the diet the activity increased. When the

content of sodium chloride in the diet was made 5% the activity of the rats was raised to a level practically as high as in adrenalectomized rats that were treated with an extract of the adrenal cortex.

Adrenalectomized rats that were maintained on a diet high in sodium chloride and low in potassium were able to perform more work, as measured by the method of Ingle, than other adrenalectomized rats given a diet of dog chow. However, the work of all adrenalectomized rats was only a small fraction of that of a normal rat (Ingle, 1940).

Toxicity of Certain Drugs and Salts. Laszt (1939) by treatment with sodium chloride was able to counteract the toxic effect and to restore the impaired intestinal absorption of glucose which follows the administration of iodoacetic acid to normal animals. Clark and Barnes (1940) confirmed this result in a striking manner. When a group of normal rats were given sodium chloride-sodium citrate in the drinking water all survived the injection of 80 mg. of iodoacetic acid but another group given water alone lived on the average only 8 hours after injection of 80 mg. of the same compound. A beneficial effect of sodium chloride was also shown when colchicine and mercuric chloride were given to normal rats compared with other rats that were given water alone or cortical extract.

Rapoport and Guest (1942) treated adrenalectomized rats with potassium and sodium phosphate and found that the lethal dose of the phosphate ion was about the same as that of potassium. However, when sodium chloride was given, the tolerance of all of the adrenalectomized rats was much increased even when the phosphate was given with a small amount of potassium ion.

Amberg and Helmholz (1919) were able to protect guinea pigs from the toxic effect of potassium chloride by the simultaneous injection of sodium chloride. The time relationships excluded the kidney from consideration as a factor and if the sodium chloride was given first in a separate solution the protection afforded against the subsequent injection of potassium chloride decreased with time. Sodium acetate did not protect the animals from the toxic effect of potassium chloride. Amberg and Helmholz described the result as an example of the antagonistic effect of sodium chloride to potassium and it is difficult to explain the experiments in any other manner.

Intestinal Response to Vasoconstrictor Influences. Fowler and Cleghorn (1942) observed that a noteworthy feature of most cats dying of adrenal insufficiency was the highly contracted state of the small intestine. When such an animal was given physiologic solution of sodium chloride by intravenous injection the response of the intestine to stimulation of the splanchnic nerve or to epinephrine was restored to normal. This was

interpreted as showing that the original contracted state was caused by a decrease of the blood volume or by the ionic ratio of sodium, potassium, and chloride in the intestine.

Metabolism of Melanin. Spoor and Ralli (1944) investigated the hides of rats in regard to the formation of melanin. The rats were given various diets and some were adrenalectomized. On a diet deficient in the filtrate factors of vitamin B there was a decrease of the extractable melanin of the skin. Dehydration by means of 2% solution of sodium chloride or by deprivation of water produced chemical changes in the skin which simulated to a certain extent those induced by a deficiency of the filtrate factor. Adrenalectomy in rats fed the deficient diet resulted in an increase of the extractable melanin. The skins of adrenalectomized rats deprived of salt but fed diets supplemented with the filtrate factors did not contain greater amounts of melanin than the skins of normal rats. The results indicated that the metabolism of melanin was influenced by both the adrenal cortex and sodium chloride.

Changes in the Anterior Pituitary Body. Koneff *et al.* (1941) summarized their investigation of the effect of sodium chloride on the anterior pituitary body of the adrenalectomized rat as follows: "If we can draw any parallel between the morphological state of a cell and its capacity to perform its specific functions, then the administration of sodium chloride to the adrenalectomized rat results to a considerable extent in the preservation of the physiological status of the anterior pituitary chromophils.

"In view of our present knowledge of pituitary physiology it seems reasonable to assume that there is little or no deficiency in the secretion of thyrotropic hormone in the salt-maintained animal. The changes in the unsupported animal, on the other hand, make adequate secretion of this hormone impossible, the inadequacy of secretion being responsible for the hypoactive thyroid seen in adrenal insufficiency."

The Estrous Cycle and Reproduction. Kutz *et al.* (1934) found that all of a group of adrenalectomized rats not given sodium chloride (Rubin-Krick solution) remained in diestrous state until death, but those given a high intake of sodium chloride maintained a normal estrous cycle. This was interpreted as a result of the general ill health of the first-mentioned group rather than because of the absence of any specific hormone.

It was also shown that after complete adrenalectomy both male and female dogs remained fertile if they were maintained with sodium chloride and sodium citrate (Kendall *et al.*, 1936).

Erythropoiesis. Crafts (1941) observed a decrease of erythrocytes in rats for an interval of 10 to 20 days after removal of the adrenal glands when the rats were maintained with 1% solution of sodium chloride.

At the end of this period the erythrocyte count and hemoglobin returned to approximately normal values.

Basal Metabolism and Uptake of Oxygen. Brownell and Hartman (1941) found normal basal metabolic rates for adrenalectomized dogs that were maintained with sodium salts.

An intimate relation between consumption of oxygen and the concentration of sodium chloride was suggested by Cohn and Soskin (1943). The consumption of oxygen by dogs after depletion of sodium chloride by an intraperitoneal injection of glucose was abnormally low and was restored to the normal value by administration of a solution of sodium chloride sufficient to increase the volume of the blood to normal. The results indicated that the favorable effect of sodium chloride was exerted in large measure by the chloride ion, and the suggestion was made that this ion is involved in the mechanism for the transport and transfer of oxygen from blood to tissue. This work was continued with the study of the uptake of oxygen by rat muscle (diaphragm) (Cohn *et al.*, 1943). It was concluded that sodium chloride influenced the rate of dissociation of oxygen and hemoglobin and thus affected the amount of available oxygen.

X. The Symptoms of Adrenal Insufficiency

The investigations of Loeb and Harrop and their associates in 1932 to 1934 definitely related the symptoms associated with removal of the adrenal cortex with the metabolism of sodium, potassium, and chloride and with the abnormal distribution of water. During the following ten years many hypotheses were suggested to explain the cause of the symptoms and from these interpretations several issues arose which were controversial. It is not my purpose to dwell on the merits of the viewpoints, but a brief review of some of the experimental results may help to clarify the situation as it exists even today.

1. Sodium, Chloride and Water

All investigators have agreed that the loss of sodium and chloride is always associated with adrenal insufficiency, but Britton *et al.* (1938) pointed out that sodium and chloride could be decreased in the blood serum of normal animals to a value much below the concentration found after adrenalectomy and yet such animals did not show the symptoms of adrenal insufficiency. Therefore it was argued that the loss of sodium and chloride was of secondary importance. Harrop (1936b) mentioned work from six different laboratories which indicated that the decrease of blood volume in adrenal insufficiency was because "the blood water

slowly transudes into the tissues and interstitial spaces and is immobilized." The loss of water was believed to depend on the "inability to maintain capillary tone and normal permeability."

A second view proposed by Darrow and Yannet (1935) assumed the withdrawal of fluid from both plasma and interstitial spaces actually into the tissue cells because of the changes of osmotic pressure which followed the loss of sodium chloride through the kidney.

Investigations which have been carried out during the past twelve years have served to strengthen this hypothesis. For man, the cat, and the dog the symptoms of adrenal insufficiency follow the loss of sodium chloride which appears to be the primary departure from normal.

The abnormal distribution both of water and of electrolytes is satisfactorily accounted for by this hypothesis, and the dramatic result of treatment with sodium chloride would seem to complete the evidence and establish the validity of the original concept of Darrow and Yannet. Further work, however, has indicated the necessity of adding another chapter which contains many paradoxes and contradictions, but the accumulated investigations of the past ten years afford convincing evidence that intimately associated with the loss of sodium chloride as a primary cause of the symptoms of adrenal insufficiency there is another departure from normal; namely, retention of potassium.

2. Potassium

The presence of potassium in the intracellular compartment in a concentration approximately 20 times that in the serum; the maintenance of the concentration of this cation within a narrow range both in the cells and in the extracellular phase; the necessity for potassium for growth; the rapid changes in the concentration of potassium in the plasma associated with metabolic processes, with the administration of certain hormones and stimulation of muscle; the effect of the concentration of potassium on the contraction of muscle as shown by the prostration resulting from familial periodic paralysis and by the beneficial effects of an infusion of salts of potassium; the influence of potassium on certain enzymes as shown by *in vitro* studies; its essential function in the propagation of the nerve impulse; and the striking evidence of the toxicity of potassium after intravenous injection—all of these aspects of the physiologic importance of potassium suggest that a disturbance of the metabolism of this cation would cause deep-seated changes that would be associated with definite signs and symptoms.

The brilliant results obtained both with patients who had Addison's disease and with adrenalectomized dogs, when given only sodium salts, tended to obscure the fact that some of the symptoms of adrenal insuffi-

ciency were directly related to potassium. The best evidence for this relationship is the administration of small amounts of potassium salts to patients who have Addison's disease or to adrenalectomized animals that have been maintained with sodium chloride and sodium citrate. The results are striking, but before these are described other relations between potassium and certain physiologic processes and the toxicity of potassium will be discussed.

Muscle. To gain quantitative evidence for the relation between the concentration of potassium in the plasma and its effect on physiologic processes, investigations have been carried out on normal animals and the information thus obtained has been used to explain the behavior of animals after adrenalectomy.

The relation between potassium and muscular activity has been studied by Miller and Darrow (1940b, 1941) who concluded that within wide limits the amount of potassium in the muscle cells of normal rats does not limit the capacity to swim continuously for 60 minutes. Concentrations of potassium in the serum that are abnormally low are not in themselves sufficient to produce "paralysis" or muscular weakness in normal animals.

Darrow (1944) made the point that "most workers on adrenal insufficiency agree that the rise in muscle potassium is not the explanation of the muscular weakness but a more or less physiological reaction to the rise in concentration of serum potassium. Indeed, the reviewer has unpublished data on rats with chronic adrenal insufficiency which show low muscle potassium while on a diet low in potassium. The evidence seems clear, therefore, that the rise in muscle potassium is not a direct effect of lack of the hormone on the muscle but an indirect result of renal failure leading to retention of potassium and depletion of extracellular electrolyte."

Heart. In a study of the electrocardiographic changes of the heart Winkler *et al.* (1938) found in the normal dog the successive concentrations of potassium in the serum necessary to produce alterations in the T wave, depression of the S-T segment, intraventricular block, disappearance of the P wave and arrest of the heart, and Winkler *et al.* (1941) determined the same relations in adrenalectomized dogs. The concentrations of potassium necessary to produce these effects were approximately the same in each series but the amount of potassium which was necessary to raise the content of this cation in the serum of the adrenalectomized dogs was only a third that required for the intact dog. The rate at which potassium was administered was found to be an important factor. Potassium was more toxic when it was administered rapidly than when it was administered slowly (Crismon *et al.*, 1943).

Toxicity. The changes in the electrocardiogram at definite concentrations of potassium in the serum both in normal and in adrenalectomized dogs indicated that the apparent toxicity of salts of potassium varied with the concentration of potassium in the serum rather than with that in the tissues. From a study of the tolerance of intact animals with a normal concentration of potassium in the muscle compared with another group in which there was a low concentration of potassium in the muscle Miller and Darrow (1940a) concluded that the toxic effects of injected potassium are directly related to the elevation of the concentration of potassium in the plasma and only indirectly to that in the muscle. Since less potassium could be removed from the plasma if the concentration in the muscle was high, it was suggested that the decreased tolerance for potassium shown by adrenalectomized animals could be explained by the high concentration of potassium in the muscle after adrenalectomy.

Zwemer (1937) suggested that potassium may be the common denominator for noxious and catabolic processes and that the increase of potassium in the plasma could be both an effect of the original stimulus and a cause of the symptoms that follow. The effects of an injection of salts of potassium on normal and adrenalectomized animals were studied and variations in the concentration of potassium in the plasma were determined (Zwemer and Truszkowski, 1936a, 1937; Truszkowski and Zwemer, 1936).

An inverse relation between the toxicity of potassium and the activity of the adrenal cortex was indicated. In the absence of the adrenal cortex tolerance to potassium was lowest and the injection of an extract of the adrenal cortex not only lowered the potassium in the blood but also protected normal animals (mice and guinea pigs) to some extent from otherwise fatal amounts of potassium administered intraperitoneally.

Schamp (1941) made strong objections to the conclusion that potassium is an important factor in adrenal insufficiency. As evidence for his contention a continuous intravenous injection of potassium chloride was made into normal conscious dogs for periods of from 2 to 12 days. No anorexia, asthenia, or convulsions were observed in any of the animals and from these data it seemed doubtful that poisoning from potassium is the primary cause of the symptoms of adrenal insufficiency in the dog.

Keith *et al.* (1942) pointed out the importance of the rate at which potassium is given. Earlier work carried out more than 100 years ago had demonstrated the toxic effect of potassium given intravenously and the absence of toxicity when a similar dose was given slowly. Large amounts of potassium salts can be ingested for the time required to reach the blood stream, the rate of excretion and the capacity of the tissues to

absorb potassium are factors which retard the accumulation of this cation in a concentration which is toxic.

Investigations on the toxicity of potassium may be divided into two groups. In the first group are studies in which a certain concentration of potassium in the serum can be related to the behavior of the heart or to survival of the animal. These concentrations of potassium in the serum are high and they obtain in adrenalectomized animals only in the terminal stages. In the second group are studies of the minimal amounts of potassium which can produce asthenia, weakness, prostration, and other symptoms of adrenal insufficiency. It is in connection with this group that controversies have arisen.

The investigations mentioned in the preceding paragraphs which come within group 2, and which were carried out on intact animals are based on an assumption the importance of which is difficult to measure. The assumption is that in the determination of the tolerance to potassium the adrenal gland of the normal experimental animal did not modify the course of the experiment. It is impossible to evaluate the degree of protection afforded by the adrenal gland of the intact animal. Valid conclusions concerning the toxicity of potassium can be made only with adrenalectomized animals that are maintained without the use of an extract of the adrenal cortex. When this was done the results indicated that the toxicity of potassium cannot be expressed in terms of its concentration in the plasma. Keith and Binger (1935) increased the concentration of potassium in the plasma of normal persons to a value higher than that observed in animals that were in a critical condition with adrenal insufficiency, but in such individuals the potassium was tolerated without untoward effect.

When adrenalectomized dogs were given a diet with high content of sodium chloride and low potassium the tolerance to potassium was decreased. After a few weeks the addition to the diet of a small amount of a potassium salt precipitated a severe prostration which terminated in death unless vigorous treatment was instituted. The least amount of potassium which was used was 0.5 g. (Kendall *et al.*, 1936; Allers *et al.*, 1936), but it was found that in the determination of the tolerance for adrenalectomized dogs it was necessary to increase the dose on each successive day (Nilson, 1937). If a constant daily dose was given the adrenalectomized dog became adapted to the intake of potassium and toxic symptoms did not appear. Adaptation to the intake of potassium occurred in the complete absence of the adrenal cortex and appeared to depend on a general systemic response.

When the adrenalectomized dog was in a state of crisis there was an increase of the concentration of potassium in the serum but this was

not sufficient to explain the severity of the prostration. Cleghorn *et al.* (1939) have confirmed and extended these observations on the toxicity of potassium.

The toxicity of potassium and the inverse relation between potassium and sodium chloride were also shown by a sudden shift in the intake of sodium chloride of an adrenalectomized dog when the diet contained a small amount of potassium. If sodium chloride was withheld from the diet the dog was prostrated and death followed within 48 hours (Kendall and Ingle, 1937).

A detailed study to explain the failure of homeostasis awaits further investigation.

Harrison and Darrow (1938) concluded that in the rat the symptoms of adrenal insufficiency were not related to changes in the distribution of water. Their results supported and amplified previous work which indicated the importance of potassium, and they pointed out that since the concentration of potassium in the cells of the intact rat is held within narrow limits the higher concentration in cases of adrenal insufficiency may be assumed to indicate important and profound changes in the muscle cell. A high intake of potassium by the rat shortened survival after adrenalectomy, and the administration of sodium chloride was followed by elimination of potassium from the blood and tissue cells and relief of the symptoms of adrenalectomy, which was just as striking as that encountered in the adrenalectomized dog with similar treatment.

The toxic effect of potassium on adrenalectomized rats was clearly shown by the use of a diet in which the content of sodium chloride was not increased but the content of potassium was greatly decreased. Wistar strain rats after adrenalectomy survived an average of 16 days on the potassium-deficient diet and an average of 11.1 days when this diet was supplemented with potassium to the normal concentration. Wisconsin strain rats after adrenalectomy survived 10.8 days on the diet low in potassium and 6.1 days when this cation was present in usual amounts (Alcott and McEwen, 1940).

Shortly after Loeb had shown the beneficial effect of sodium chloride during adrenal insufficiency Rubin and Krick (1934) suggested the use of a solution to replace tap water for treatment of adrenalectomized rats. They found from balance studies for sodium, potassium, calcium, magnesium, chlorine, phosphorus, and nitrogen that adrenal insufficiency was associated with a loss from the body of all the elements investigated. Since treatment should attempt replacement of these losses a solution of 0.7% sodium chloride, 0.035% potassium chloride, 0.0329% calcium chloride, and 0.015% magnesium chloride was substituted for the drinking water. The results were satisfactory. Adrenalectomized rats were

maintained in apparent health for 4 months with this mixture, and since that time the Rubin-Krick solution has been widely used by investigators in this field.

More recent work suggests a higher intake of sodium chloride (Anderson, 1943) and a word of caution may be in order concerning the intake of potassium. If potassium is provided in the drinking water the amount in the diet should be reduced, but since it is easier to use a diet with a normal content of potassium the need or advantage of addition of potassium to the drinking water may be questioned.

In order to separate the influence of the adrenal cortex on the tissues, quite apart from the changes induced by loss of electrolytes and water through the kidney, Ingle *et al.* (1937) determined the effect of an extract of the adrenal cortex on nephrectomized-adrenalectomized rats. The extract did not change the concentration of sodium chloride in the blood nor prevent the rise of urea, but the increase of potassium was delayed and the normal capacity of the muscle to work was maintained. The loss of muscular activity was associated with an increase of the concentration of potassium in the plasma. It was believed that the effect of the extract was concerned with its favorable effect on the distribution of potassium.

MacKay *et al.* (1937) confirmed the observations on nephrectomized-adrenalectomized rats and suggested that the most likely explanation for the result was the retention of some "toxic substance" which does not reach the serum in the presence of the adrenal glands, or is excreted in the urine when the kidneys but not the adrenals are present. They did not think that the "toxic substance" was potassium. However, it was later shown by Durlacher and Darrow (1942) that if the tissues of rats were depleted of potassium by a diet with low content of potassium, survival was lengthened after nephrectomy or ureteral ligation. There was a delay in the increase of potassium in the serum from an abnormally low value toward normal values and this delay was attributed to an uptake of potassium by the muscle. The longer survival was attributed to the delay in the rise of potassium in the plasma to toxic levels.

XI. Qualitative Differences of Steroids

1. *The Sodium Factor*

Hartman and Spoor (1940) have separated a fraction from an extract of the adrenal cortex which appeared to have a specific effect on the retention of sodium, but despite continued work (Hartman and Thatcher, 1946) the chemical nature of this material has not been determined. Production of a refractory state suggests some type of sensitization to protein. Retention of sodium in normal and adrenalectomized animals

has been used as a method of standardization (Hartman *et al.*, 1941) but the use of this criterion was not recommended by Olson *et al.* (1944).

2. The Amorphous Fraction

The greatest influence on the metabolism of electrolytes is shown by the so-called amorphous fraction after separation of corticosterone and related compounds in crystalline form. Wells and Kendall (1940a) showed that when enormous doses of this fraction were given to normal rats an increase of the concentration of sodium and chloride in the serum and loss of potassium beyond the average values for intact rats did not occur. Prolonged administration of large amounts of this material did not retain sodium and chloride or produce edema.

The chemical properties of the amorphous fraction indicate the presence of an unsaturated ketone and it is probable that the active agent is a steroid. The amorphous fraction is characterized by its solubility in water, which is approximately 60 times greater than that of corticosterone. This may depend on the presence of hydroxyl groups or combination with a water-soluble moiety. Lowenstein and Zwemer (1946) suggested such a possibility.

3. Estrogens, Androgens and Related Compounds

The influence of certain steroids on the metabolism of electrolytes has been clearly shown. Talbot *et al.* (1940) found that the primary change in the uterus following an injection of estradiol was an increase of water and extracellular electrolytes. There was also an increase of the ratio of potassium to phosphate but during the following 24 hours there was rapid growth of new protoplasm and the concentration of electrolytes returned to normal values. Thorn and Engel (1938) were able to show that retention of sodium and chloride was produced by estrone, estradiol, progesterone, and testosterone propionate. Kinsell *et al.* (1942) postulated that when the cardiac glucosides are administered in small amounts their influence on the potassium in the plasma is similar to that of the hormones of the adrenal cortex.

Wood and Moe (1942) found a positive correlation between the total dose of a digitalis glucoside and the rate of increase of potassium in the serum.

Zwemer *et al.* (1940) observed similarities between the physiologic effects of the hormones of the adrenal cortex and that of some cardiac glucosides.

However, these modifications in the distribution of electrolytes are of secondary importance to the control which is exerted over the metab-

olism of sodium, potassium, and chloride by some of the steroids separated from the adrenal cortex.

4. Desoxycorticosterone

Sodium and Chloride. This 21-hydroxyl derivative of progesterone has been isolated in small amount from an extract of the adrenal cortex (Reichstein and von Euw, 1938) but the results of both chemical and physiologic investigations suggest that only traces of this compound are in the gland. The favorable effect of desoxycorticosterone on adrenalectomized dogs was first reported (Thorn *et al.* 1938) shortly after the partial synthesis of this compound had been completed (Steiger and Reichstein, 1937). Even with a low intake of sodium chloride Thorn and Eisenberg (1939) maintained an adrenalectomized dog in good condition with 1.5 mg. desoxycorticosterone acetate/day, and Cleghorn *et al.* (1941) showed that the daily dose of the compound varied inversely with the intake of sodium chloride. Desoxycorticosterone acetate given in small daily doses maintained life and growth in adrenalectomized rats when they were given purified diets virtually free of sodium chloride (Eversole, 1945).

The administration of desoxycorticosterone acetate not only stopped the loss of sodium and chloride in the adrenalectomized dog but in addition caused a marked retention of these electrolytes (Thorn *et al.* 1938).

Associated with the increase of these electrolytes in the extracellular phase there was an increase of the volume of the plasma and of the arterial pressure (reviewed by Swingle and Remington, 1944). Kuhlmann *et al.* (1939) showed that desoxycorticosterone acetate produced a marked disturbance of the metabolism of water and sodium chloride in normal dogs. Daily administration of 20–25 mg. of the steroid produced polydipsia and polyuria. Ragan *et al.* (1940) found an increased concentration of sodium and chloride in the plasma. The addition of sodium chloride to the drinking water resulted in a further increase of the intake of water and output of urine. At the same time the specific gravity of the urine was decreased. Restriction of water was followed by a sharp rise of the concentration of sodium in the serum and an increase of the specific gravity of the urine. When water was again freely allowed, the intake increased greatly, the sodium in the serum decreased and the specific gravity of the urine was less.

When the administration of desoxycorticosterone acetate was stopped, the polydipsia and polyuria disappeared even when the administration of sodium chloride was continued. Readministration of desoxycorticosterone acetate resulted in reappearance of the entire syndrome. Although the condition superficially resembled that of diabetes insipidus,

the two states were dissimilar in at least two important respects: solution of posterior pituitary was relatively ineffective and restriction of fluid did not cause dehydration. It was suggested that the most important abnormality was a thirst and secondarily a polyuria. Whether the thirst was related to the increase of sodium in the extracellular phase or to some other cause was not clear, but it was evident that the resulting polyuria enabled the normal dog to avoid excessive retention of sodium. The rapid flow of urine through the tubules would tend to counteract the increased reabsorption of sodium and chloride brought about by the desoxycorticosterone acetate.

Richter (1941) showed that in adrenalectomized rats treated with desoxycorticosterone acetate the appetite for sodium chloride decreased to the normal level. The intake of water decreased sharply during treatment and increased sharply after discontinuation of treatment. The intake of water closely followed the intake of sodium chloride.

Rice and Richter (1943) observed a different effect of desoxycorticosterone acetate in normal rats. It was found that under the influence of the steroid there was an increased appetite for salt and an associated polydipsia and polyuria. The polydipsia appeared to be a direct consequence of the high intake of sodium chloride rather than a manifestation of a primary effect on the metabolism of water.

Winter and Ingram (1943) found that in normal dogs desoxycorticosterone acetate in large doses increased glomerular filtration and reduced the reabsorption of water by the tubules. These investigators also reviewed the suggestions which have been made to explain the relation between the adrenal cortex and the pituitary body in adrenal insufficiency and in diabetes insipidus.

Potassium. Thorn *et al.* (1938) found an increase of the excretion of potassium when desoxycorticosterone acetate was given to normal dogs, and in 1939 Kuhlmann *et al.* described the striking effect of the administration of large amounts of this compound to normal dogs. There was a decrease of the concentration of potassium in the serum to values much less than normal and the dogs showed a curious periodic weakness with inability to stand or raise the head. These bouts of paralysis, except for persistence of reflexes, closely resembled familial periodic paralysis in man, which is also associated with marked decrease of potassium in the serum.

Ferrebee *et al.* (1941) extended the observations made on the loss of potassium from serum and muscle and the beneficial effect of an injection of potassium chloride on the symptoms associated with the paralysis. It was found that the ingestion of potassium chloride prevented loss of potassium from the muscle and the periodic development of weakness but the higher intake of potassium did not significantly influence either

the "diabetes insipidus" or the elevated concentration of sodium in the serum. It was therefore suggested that the "diabetes insipidus" was associated with concentration of sodium in the serum, and the muscular weakness with the concentration of sodium and potassium within the muscle cells.

Truszkowski and Duszynska (1940) reported some degree of protection afforded to mice by desoxycorticosterone acetate against the toxic action of injections of potassium chloride. For example, the L.D. 50% for normal mice weighing 9–10 g. was found to be 6.65 mg. potassium chloride/10 g. body weight. That for adrenalectomized mice was 5.75 mg./10 g. body weight, and for normal mice after injection of 1 mg. of desoxycorticosterone acetate it was 7.7 mg.

The results produced by desoxycorticosterone acetate in normal and adrenalectomized animals both on the retention of sodium and on the excretion of potassium are in strong contrast to those obtained by the administration of an extract of the adrenal cortex (Wells and Kendall, 1940a). This constitutes biologic evidence that desoxycorticosterone is not an important constituent of the secretion of the adrenal cortex.

5. Corticosterone and Related Compounds

The hormones of the adrenal cortex with a ketone or hydroxyl group at C_{11} and with hydrogen at C_{17} have but slight effect on the retention of sodium and chloride or on the excretion of potassium (Thorn *et al.*, 1938; Wells and Kendall, 1940a; Thorn *et al.*, 1941; Ingle *et al.*, 1946).

The two hormones with a ketone or hydroxyl group at C_{11} and with a hydroxyl group at C_{17} were found to increase the excretion of sodium and chloride in a normal dog (Thorn *et al.*, 1941). However, it was shown by Ingle *et al.* (1945, 1946) that the loss of sodium and chloride in the rat was but temporary and the normal balance was quickly restored.

The excretion of potassium was increased when a moderate amount of 17-hydroxycorticosterone was injected.

XII. Electrolytes and Adrenal Insufficiency in Clinical Medicine

1. Diagnosis

Loeb *et al.* (1937) showed that of the three important changes in a group of patients who had Addison's disease the one most frequently encountered was a decrease of the concentration of sodium in the serum. An increase of the value for potassium and an increase of nonprotein nitrogen were second and third respectively.

Harrop *et al.* (1933c) proposed a test for the diagnosis of Addison's

disease based on withholding sodium chloride from the diet of a patient for six days. However, at that time the importance of the antagonism between sodium and potassium was not realized. Wilder *et al.* (1937) showed that a patient who had adrenal insufficiency could withstand the effects of a low intake of sodium salts if the intake of potassium was also low, but if the intake of potassium was slightly increased the loss of sodium and chloride in the urine was increased and symptoms of adrenal insufficiency developed.

These observations suggested a modification of Harrop's test which was devised by Cutler *et al.* (1938) and extended by Willson *et al.* (1942). The intake of sodium chloride was limited and moderate amounts of a potassium salt were given for a period of 52 hours. Under these conditions it was found that in patients who had adrenal insufficiency the concentration of sodium and chloride in the urine during the last 4 hours of the test was so much greater than that of normal controls that the results provided a diagnostic procedure which was highly reliable.

Experience with this test disclosed the fact that the group of patients who excreted a high concentration of sodium and chloride also showed inability to excrete water in a normal manner. The volume of the urine excreted during certain intervals of the test provided information which was almost as reliable as that given by the 52-hour test (Robinson *et al.*, 1941; Levy *et al.*, 1946).

Summarizing the results of an extended study of the relation between the adrenal cortex and water diuresis and the bearing that these results have on the value of clinical diagnostic tests Gaunt (1946) expressed the following opinion: " . . . it is safe to conclude that there are no important exceptions to the rule that adrenal insufficiency, even in a relatively mild state, is associated with a decreased diuretic response to water, when water is ingested in sizable dosage."

With the results on experimental animals as a basis, Zwemer and Truszkowski (1936b) suggested a clinical test for the function of the adrenal cortex which consisted in ingestion of a salt of potassium to yield 10 mg. of potassium/pound body weight. This amount of potassium did not increase the concentration of this ion in the serum of a normal person but in cases of Addison's disease the concentration was increased. Further investigation, however, has shown that the concentration of potassium in the serum cannot be used as an index for the activity of the adrenal cortex (Greene *et al.*, 1940).

2. Treatment with Control of Electrolytes

Wilder *et al.* (1937) extended the experimental work that had established conditions satisfactory for the maintenance of adrenalectomized

dogs and showed that the same regimen was equally suitable for patients who had Addison's disease. A high intake of sodium chloride and sodium citrate was essential and it was also found that the condition of the patient was in large part dependent on the intake of potassium. As in the adrenalectomized dog the inverse relation between sodium and potassium was clearly evident. When the intake of potassium was low the intake of sodium salts could be reduced but if the intake of potassium was increased even to the amount contained in an ordinary diet sodium and chloride were rapidly lost and symptoms of adrenal insufficiency developed. With adequate control of the electrolytes the patients enjoyed a sense of well-being and carried out their usual activities indefinitely without the need of substitution therapy with an extract of the adrenal cortex.

However, the lives of these patients were in a precarious condition. Overindulgence in food with a high intake of potassium, a gastrointestinal upset, infections of the respiratory tract, a psychic strain or physical trauma, in short, anything that interfered with ingestion of sodium chloride and sodium citrate at regular intervals was a potential threat. As long as the intake of water and electrolytes remained in balance the patients were well, but in the event of a crisis the only recourse was administration of an extract of the adrenal cortex. When desoxycorticosterone became available this situation was relieved.

3. *Treatment with Desoxycorticosterone Acetate*

Thorn *et al* (1939) administered desoxycorticosterone acetate to patients who had Addison's disease and noted a positive balance of sodium and chloride, an increase of the concentration of these ions in the serum, an increased renal excretion of potassium, and an increase of the volume of the plasma, the blood pressure, and the body weight.

During the first use of desoxycorticosterone acetate it was not realized how much this compound would influence the retention of sodium and chloride. Thorn and Firor (1940) suggested that "patients with Addison's disease can be treated successfully either with desoxycorticosterone acetate alone or with this factor supplemented by sodium chloride therapy." However, they also warned that "it is evident that the quantity of supplementary sodium chloride administered to such patients must be carefully regulated during treatment with desoxycorticosterone acetate. Excessive quantities of the synthetic adrenal cortical principle and sodium chloride may result in the appearance of edema, hypertension, and in some instances signs of congestive heart failure."

It was further found that the continued administration of excessive quantities of desoxycorticosterone acetate and sodium chloride may

result in an abnormal decrease of the concentration of potassium in the serum, which in some patients was associated with muscular weakness and transient paralysis. The administration of potassium citrate was recommended to overcome the loss of potassium and restore normal muscular activity.

Ferrebee *et al.* (1939) concluded that the improvement of the condition of the patient during treatment with desoxycorticosterone acetate was qualitatively identical with that following the administration of salt and water alone, but quantitatively the improvement was far more marked.

These investigators warned against the use of salt beyond the amount in the usual diet because of the increase of the blood volume.

Tooke *et al.* (1940) also drew attention to the fact that when desoxycorticosterone acetate was used the retention of sodium and chloride and the excretion of potassium were so marked that the use of diets with the content of sodium chloride high and that of potassium low was not necessary. Von dem Borne and Lopes Cardozo (1940) warned against overdosage of desoxycorticosterone acetate, particularly with a high intake of salt.

XIII. Comment and Summary

The preceding sections of this review contain the evidence that the metabolism of sodium, potassium, and chloride is in large measure controlled by the hormones of the adrenal cortex. It is also true that the physiologic response to administration of the hormones of the adrenal cortex is in large measure determined by the amount of sodium and chloride present in the body. Harrison and Darrow (1938) observed that " . . . reduction of sodium in extracellular fluids reduces the effectiveness of the hormone. This fact was demonstrated in the experiments in which adrenalectomized rats were further depleted of sodium by intraperitoneal injection of glucose solution. It had previously been found that 5 cc. of cortical extract sufficed to produce a rapid cure of all symptoms in rats which had been allowed to progress to the terminal stages of insufficiency. However, in the adrenalectomized rats further depleted of sodium chloride, the same amount of extract merely protected the animals from prostration and immediate death since symptoms such as diarrhea and muscular weakness persisted and the blood nonprotein nitrogen remained elevated. Furthermore, muscle potassium was not restored to normal as was always the case with adrenalectomized rats not further depleted of sodium and chloride."

Briefly stated, the adrenal cortex furnishes a mechanism to the body which modifies the transfer of sodium, potassium, and chloride through

cellular structures. In a normal animal deprived of salt the transfer of sodium and chloride from the glomerular filtrate to the blood against osmotic pressure until almost no sodium or chloride remains in the urine is perhaps the best example of the essential action of the adrenal cortex. This, however, is only one example. The transfer of ions through the intestinal wall, the mobilization of sodium and chloride in the extracellular compartment after depletion of these electrolytes by intraperitoneal injection of glucose, the recovery from "water intoxication" and the restoration of strength and vigor to an adrenalectomized dog that shows the typical symptoms of adrenal insufficiency solely through the administration of the hormones of the adrenal cortex without the aid of water or of a solution of sodium chloride (Swingle *et al.*, 1934c, 1936b, 1937b) are other processes which appear to depend on the same type of mechanism.

In man, the dog, or the cat in adrenal insufficiency an increase of the intake of sodium is followed by an increase of the output of potassium. The reverse is also true, but in the intact body an increase of the intake of either sodium or potassium causes but a brief increase of the output of the other ion and the normal balance is promptly restored. It would therefore seem that the hormones of the adrenal cortex modify the transfer of electrolytes in the kidney and permit this organ to perform work to overcome the direction of the ionic changes which occur in the absence of the adrenal cortex.

The remarkable effect of sodium chloride on renal function depends on something more than restoration of blood volume and of the normal concentration of electrolytes. Maintenance of renal function with sodium chloride seems of itself to have an important part in the quality of the response. However, in the absence of the adrenal cortex it is necessary to provide a high intake of sodium and chloride and thus insure a continuity of the supply of these electrolytes for the kidney. It is evident that both the conservation and continuity of supply of these essential ions are afforded by the mechanism which is furnished by the adrenal cortex.

At the present time the experimental work is still incomplete and the details of the mechanism by which electrolytes pass through cellular structures is not known. The next step appears to be progress in the field of organic chemistry, for it is only through the use of new tools that progress can come.

The availability of desoxycorticosterone has simplified the problem in several respects. In the first place it substitutes a single pure compound for the complex extract of the adrenal cortex. As a result the physiologic response is confined almost wholly to the changes in the

metabolism of sodium, potassium, and chloride and is not complicated by the influence of the C_{11}-oxygenated hormones which strongly modify the metabolism of fat, carbohydrate, and protein. Secondly, the effect of desoxycorticosterone acetate on the distribution of electrolytes and of water is of much greater magnitude than is that of any other known steroid or extract of the adrenal cortex. Finally, desoxycorticosterone has the smallest number of functional groups and therefore has the simplest possible chemical structure.

The problem can be defined at least in part in terms of organic chemistry, and although at the present stage of the investigation any suggestion must be clearly labeled as speculation, a suggestion may help to direct attention to the apparent heart of the problem. The ketol group attached to C_{17} has been shown to be essential for the physiologic action which is inherent in desoxycorticosterone. It is possible that the ketol group, either directly or indirectly, may affect the transport of ions through cellular membranes and in this way control the distribution of electrolytes and therefore of water.

Stewart (1924) pointed out the ghost of the detoxication theory that walked through laboratories and spread confusion. This review closes with reference to a mechanism for the transport of ions by living cells. The nature of this mechanism is as elusive as a gremlin, but when it is understood we shall know how a simple group on the steroid nucleus confers physiologic freedom from domination by the electrolytes.

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Cumulative Index of Vols. I–V

C

E

H

K

L

M

T

U

W

X

Author Index

Names in parentheses indicate senior authors of the references and are included to assist in locating references where a particular name is not on a given page. The numbers in parentheses are reference numbers; those in italics refer to the pages on which references are listed in bibliographies at the end of each article.

Examples: Abbott, W. E., 284 (see Mellors) means that Mellors *et al.* will be mentioned on page 284, the *et al.* accounting for Abbott. This article can be located under Mellors in the list of references.

Abdel-Salaam, A., 166 (ref. 1) means that reference number 1 is mentioned on page 166, without the author's name.

B

C

G

I

J

N

T

U

V

Y

Subject Index

N

O

P

Q

R